

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

Metabotropic Glutamate Receptor 5 Is a Disulfide-linked Dimer*

(Received for publication, August 16, 1996)

Carmelo Romano†§1, Wan-Lin Yang§, and Karen L. O'Malley§

From the Departments of †Ophthalmology and Visual Sciences and §Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110

The sequences of the metabotropic glutamate receptors (mGluRs) show little homology with other members of the G protein-coupled receptor family and exhibit several distinctive features, including a large N-terminal extracellular domain with 17 cysteines in conserved positions. Here we demonstrate that mGluR5, as well as other mGluRs, behave as species approximately twice as large as expected from their sequence, but reducing conditions cause a decrease to the predicted molecular mass. Co-immunoprecipitation experiments using wild type and epitope-tagged receptors demonstrate that this is due to specific, disulfide-dependent dimerization of the receptor. The intermolecular disulfide that mediates dimerization occurs in the extracellular domain, within about 17 kDa from the N terminus.

Glutamate is the primary neurotransmitter for excitatory neurotransmission in the vertebrate central nervous system and as such is responsible for a broad range of physiological and pathophysiological roles. These include transmission in sensory pathways, higher brain functions such as learning and memory, and cytotoxicity and neuronal death.

Two classes of receptors for glutamate are present on neural cells: the ionotropic glutamate receptors (iGluRs)¹ and the metabotropic glutamate receptors (mGluRs). The iGluRs are ligand-gated cation channels, and they mediate rapid synaptic transmission. The iGluRs include the *N*-methyl-D-aspartate, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, and kainic acid families of receptors. At least eight mGluRs have been molecularly characterized, and these activate effectors via interactions with heterotrimeric G proteins (1). Thus, the mGluRs are important for neuromodulatory functions, although mGluRs clearly mediate transmission at the retinal photoreceptor-depolarizing bipolar cell synapse (2–4) and at certain thalamic sensory neurons (5).

Although the mGluRs possess seven transmembrane domains, there are important differences between these receptors and other G protein-coupled receptors. There is no primary sequence similarity between the mGluRs and the rhodopsin-like receptors (4). The recently described Ca^{2+} -sensing recep-

tors are homologous to the mGluRs (6, 7); so together the Ca^{2+} and glutamate receptors appear to constitute a unique subgroup of this supergene family. Receptor domains responsible for signal transduction also appear different. For example, the third intracellular loop is important for determining the specificity of G protein coupling in most G protein-coupled receptors examined, whereas the C-terminal end of the second intracellular loop is critical in the mGluRs (8–10). Moreover, all mGluRs have a very large N-terminal extracellular domain (about 65 kDa in mGluR5), constituting about one-half of the protein, whereas most G protein-coupled receptors do not. The glutamate binding domain is believed to lie in this extracellular region (11, 12), not within the bundle of membrane-spanning domains, as is typical of the rhodopsin-like receptors.

Another unique structural feature is that there are 21 conserved cysteine residues in all the mGluRs (13). Nineteen of these are in the N-terminal domain and extracellular loops. Nine of the cysteines are at the C-terminal portion of the extracellular domain, and this region has been compared with similar cysteine-rich domains of receptor tyrosine kinases (12). Although the function of these cysteines is unknown, the strict conservation of position implies that the function is a shared and important one for this family of receptors.

Although native iGluRs are thought to function as heteromeric pentamers (14–16), the mGluRs, by analogy with other G protein-coupled receptors, have been assumed to be monomeric (1, 4, 17). However, this has not been directly demonstrated. Using biochemical and molecular techniques, here we demonstrate that mGluRs are not monomeric but are instead covalently linked dimers, bound by disulfide bonds between conserved cysteines in the N-terminal extracellular domain.

EXPERIMENTAL PROCEDURES

Antibodies, Western blots, and Immunoprecipitates—Antibodies to wild type (wt) mGluR5 were affinity-purified antipeptide antibodies raised against an immunogen that contained the C-terminal 13 amino acids as described (18). Antibodies to wt mGluR1a were affinity-purified antipeptide antibodies raised against an immunogen that contained the sequence of residues 1116–1130 (i.e. EFVYERGN-TEEDEL) of the rat mGluR1a (19). Both monoclonal and polyclonal anti-hemagglutinin (HA) antibodies were obtained from Babco (Berkeley, CA); the polyclonal antibody was used for immunoprecipitation, the monoclonal antibody for Western blots and immunocytochemistry. Antibodies to mGluR2–3 and mGluR4 were the generous gift of Dr. Thomas Knoepfel (CIBA, Basel, Switzerland). Preparation of brain tissue, electrophoresis (on 6 or 7.5% polyacrylamide gels), and transfers onto polyvinylidene difluoride membranes (Immobilon P; Millipore, Waters, MA) were as described (18). For preparation of membranes from transfected cells, cells were washed once in PBS, then subjected to one freeze-thaw cycle. They were scraped into lysis buffer (2 mM HEPES and 2 mM EDTA, pH 7.4, containing protease inhibitors) and homogenized in a glass homogenizer with a motorized Teflon pestle. The nuclear pellet (1000 \times g, 5 min) was discarded, and membranes were harvested after pelleting (35,000 \times g, 30 min). For immunoprecipitations, membranes were homogenized in PBS containing 0.5 or 1% SDS. The SDS extract was diluted 5- or 10-fold into PBS containing 0.5% dodecyl maltoside to sequester free SDS into mixed micelles, thereby permitting immunoprecipitation. Antireceptor antibody was added, and

* This work was supported in part by National Institutes of Health Grants AG11355, EY02687, EY09370, an unrestricted grant from Research to Prevent Blindness, and the McDonnell Center for Cellular and Molecular Neuroscience. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Ophthalmology and Visual Sciences, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-2676; Fax: 314-362-3638; E-mail: romano@am.seer.wustl.edu.

¹ The abbreviations used are: iGluR, ionotropic glutamate receptor; mGluR, metabotropic glutamate receptor; wt, wild type; HA, hemagglutinin; tHA, truncated HA; PBS, phosphate-buffered saline; DTT, dithiothreitol; BPB, periplasmic binding protein.

the mixture was incubated at 4 °C overnight. Protein A-Sepharose (Sigma) was added, and the incubation continued for 2 h at room temperature on a rocking table. The protein A pellets were washed three times with PBS before elution with sample buffer and electrophoresis. The sample buffer always contained 2% SDS and, when indicated, 20 mM dithiothreitol (DTT). Samples were heated at 60 °C for 3 min before electrophoresis.

Receptors and Cells—The cDNA fragments containing the full-length mGluR5 or mGluR1a coding sequences were ligated into pcDNA1neo (Invitrogen) downstream of the cytomegalovirus promoter (18). The HA-tagged mGluR5 mutant was constructed using recombinant polymerase chain reaction (20) with two sets of primer pairs. The first set (5'-CATGACGACCTTCGAGAGAT-3', nucleotides 3482 to 3501, GenBank accession number D10891; and 5'-ATCCTCTCCAAATATGACACTTATCATATGATGTTCCAGATTATGCTT-3', nucleotides 3720–3741 followed by the HA epitope in bold) and the second (5'-TATCCATATGATGTTCCAGATTATGCTTGGAGCCACTGGAACTTCCT-3', representing the HA epitope in bold followed by nucleotides 3781–3801; and 5'-CACACACGGTGGAGACATGAGCGGCCGCTAAA, nucleotides 3897–3918 followed by a *NotI* restriction site) were used to amplify DNA fragments of 286 or 164 base pairs from wt-mGluR5. Both fragments were used as templates in another round of polymerase chain reactions using the two flanking primers, and the resulting fragments were then used to replace wt sequences in mGluR5. tHA was constructed using HA-tagged mGluR5 as a template together with primer sets fusing sequences within the first intracellular domain in frame with those of the HA-tagged C terminus. The 5'-primer set included 5'-GAAGTCAGCTGTTGTTGG-3' (identical to nucleotides 1843–1860) as well as 5'-CGAGTCCACCGAGTCTCTAGACTTGACACCGGAGT-3' (complementary to nucleotides 2083–2097 and 3685–3668). The 3'-primer set consisted of the complement of the latter primer together with the *NotI* restriction site-containing primer described above. First and second round polymerase chain reaction was performed as described. The final product was digested with *BstEII* and *NotI* and then subcloned into wt-mGluR5 cut with the same enzymes. The resulting plasmid was termed tHA. Both the HA-tagged mGluR5 and tHA were confirmed by sequencing.

HEK cells at 80% confluency were transfected with 15 µg of plasmid DNA using LipofectAMINE (Life Technologies, Inc). Forty-eight hours later membranes were prepared and immunoprecipitated with the indicated antibodies as described above. Immunohistochemical analysis of cotransfected cells was done on Lab-Tek chambered glass slides. Primary antibodies included the HA monoclonal antibody and anti-wt-mGluR5, followed by fluorescein isothiocyanate-labeled goat anti-mouse and CY3-labeled goat anti-rabbit (Jackson ImmunoResearch Labs, Inc.) secondary antibodies, respectively.

RESULTS

Metabotropic Glutamate Receptors Migrate at about Twice Their Predicted Molecular Mass under Nonreducing Conditions—To examine the possible structural or conformational roles of the conserved cysteines in mGluRs, the electrophoretic mobility of mGluR5 (from rat cortical membranes) was examined under reducing and nonreducing conditions in SDS gels (Fig. 1A). In the presence of the reducing agent DTT or 2-mercaptoethanol (not shown), mGluR5 migrated at an apparent molecular mass of ~148 kDa. Deglycosylation with peptide N-glycosidase F reduced this to ~130 kDa (not shown), consistent with the size predicted from the primary sequence. However, in the absence of reducing agent, mGluR5 migrated at an apparent molecular mass of ~260 kDa (Fig. 1A). Because samples were prepared in the presence of the denaturing detergent, 2% SDS, most noncovalent interactions should have been eliminated.

One interpretation of this result is that mGluR5 is covalently attached by intermolecular disulfide bonds to another component of the membrane, but other possibilities must be considered. A different electrophoretic mobility in the presence of reducing agents may reflect an altered conformation due to cleavage of intramolecular disulfide bonds. However, such bonds usually promote compact structures that on reduction increase the Stokes radius and, hence, increase the apparent molecular mass of the protein. Another possibility is that spu-

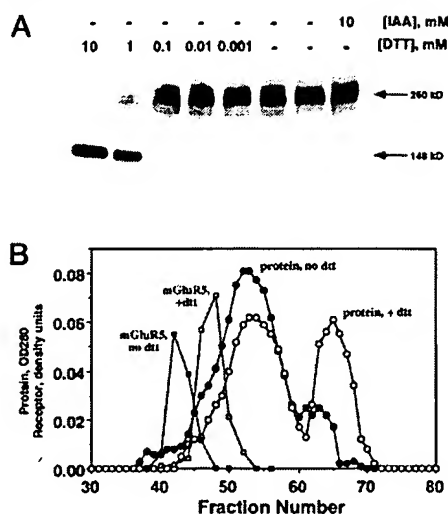


FIG. 1. mGluRs have a high apparent molecular mass under nonreducing conditions. A, Western blot analysis of rat cortical membranes (25 µg of protein/lane) using an antibody directed to the C terminus of wild type mGluR5. DTT caused the receptor to shift to a lower apparent molecular mass. Pretreatment of the membranes with iodoacetate (IAA) did not alter the apparent molecular mass. B, Sephacryl S-400 column chromatography of SDS-solubilized rat brain membranes in the presence or absence of DTT indicate that mGluR5 migrates as a lower molecular mass species in the presence of DTT. Protein was measured spectroscopically (OD280), and mGluR5 was detected by assaying every other fraction by Western blotting. Column dimensions, 75 × 1.5 cm.

rious disulfide bonds may form between free sulfhydryls during SDS denaturation of the protein, leading to artifactual covalent association of mGluR5 with another protein. To test this, membranes were treated with iodoacetate to alkylate free sulfhydryls and then solubilized with SDS (Fig. 1A, right lane). The receptor still behaved as a high molecular mass species, indicating that the disulfide bonds responsible for holding this species together were present prior to solubilization, as part of the native structure (Fig. 1A).

To verify this reduction-dependent alteration in molecular mass by an independent technique, Sephacryl S-400 gel filtration chromatography in the presence of SDS was used. For this experiment cortical membranes (2 mg of protein) were dissolved in PBS containing 1% SDS (± 10 mM DTT), which also served as column buffer. Reduction led to a large decrease in apparent molecular mass of the receptor (Fig. 1B). Taken together, these results indicate that mGluR5 is covalently attached via disulfide bonds to another component(s) of the membrane.

This molecular mass shift was not unique to mGluR5. Under nonreducing conditions mGluR1a (another group 1 mGluR), mGluR2–3 (a Group 2 mGluR) and mGluR4 (a Group 3 mGluR) all migrated as species about twice as large as expected, with reduction causing a shift to the appropriate molecular mass (data not shown).

Metabotropic Glutamate Receptors Are Dimers under Nonreducing Conditions—What is the nature of the molecule to which mGluR5 is attached? The receptor may be bound by disulfide bridges to a distinct molecule; therefore, the high molecular mass species would be heteromeric, or alternatively, the receptor may be a homodimer.

When mGluR5 was expressed in HEK cells, it migrated as the high molecular mass form in nonreducing gels (not shown); this indicated that either the receptor forms homodimers, or that HEK cells endogenously express the mGluR5-associated protein. To determine which of these hypotheses is correct,

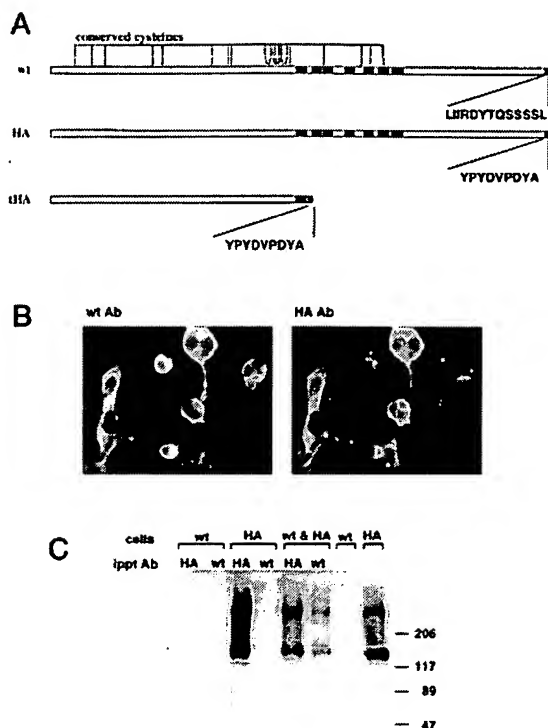


FIG. 2. Co-immunoprecipitation of wt and epitope-tagged receptors. *A*, schematic representations of wt, HA-tagged, and tHA mGluR5 receptors. Putative transmembrane domains are shown in black, and positions of the 21 conserved cysteine residues are indicated. The amino acid sequences of wt and HA epitopes present at the C termini of the receptors are shown. *B*, HEK 293 cells co-transfected with both HA-tagged and wt mGluR5, labeled with antibodies (Ab) to wt (left panel) or HA-tagged (right panel) mGluR5. The same population of cells are labeled. *C*, mGluRs form dimers. Membranes from cells expressing HA-mGluR5, wt-mGluR5, or both were treated with SDS and immunoprecipitated with HA polyclonal or wt antibodies (ippt Ab). Precipitated products were reduced using 20 mM DTT and resolved on a 6% SDS-polyacrylamide gel. Separated products were transferred to a polyvinylidene difluoride membrane and probed with anti-HA monoclonal antibody followed by enhanced chemiluminescence. Molecular mass markers are shown on the right. Antibody to the wt receptor immunoprecipitated HA receptor from cotransfected cells, indicating that heterodimerization occurred.

cross-immunoprecipitation experiments were performed. A plasmid encoding an mGluR5 epitope tagged at the C terminus was constructed. Because the antibody we have used to recognize wt mGluR5 is directed toward the C terminus (18), the nucleotides coding for the wt C terminus were removed and replaced by the sequence encoding the HA epitope (Fig. 2A; Ref. 21). After expression in HEK cells, HA-mGluR5 also behaved as a high molecular mass species in nonreducing gels, indicating that alteration of the C terminus did not disrupt formation of the disulfide-bound complex (not shown). If the receptors form covalent homodimers, some of the wt-mGluR5 and HA-mGluR5 may be expected to be found in the same dimer when both receptors are expressed in the same cell. If, however, each receptor is bound to an unidentified, distinct molecule to form the high molecular mass form, no wt-mGluR5-HA-mGluR5 heterodimers should be found.

Transient transfection of HEK cells with both plasmids led to uptake and expression of both receptors in the same individual cells (Fig. 2B). Membranes prepared from the cotransfected cells were solubilized in 0.5% SDS to disrupt noncovalent interactions between proteins. As expected, anti-wt did not immunoprecipitate any HA-tagged proteins from cells expressing only HA-mGluR5 (Fig. 2C), nor did anti-HA bring down wt-

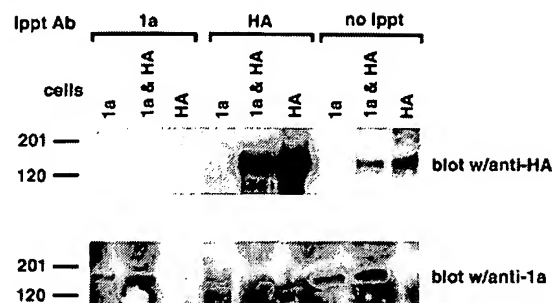


FIG. 3. mGluR1a and mGluR5 do not form heterodimers. Membranes from cells expressing wt-mGluR1a (1a), HA-mGluR5 (HA), or both (1a & HA), were treated with SDS and immunoprecipitated with anti-mGluR1a (1a) or anti-HA (HA) polyclonal antibodies. Precipitated products were reduced using 20 mM DTT and resolved on a 6% SDS-polyacrylamide gel. Separated products were transferred to a polyvinylidene difluoride membrane, and the blots probed with anti-HA monoclonal (top blot) or anti-mGluR1a polyclonal (bottom blot) antibodies followed by enhanced chemiluminescence. Molecular mass markers are shown on the left (201 and 120 kDa). Antibody to the mGluR1a precipitated mGluR1a, but not HA-mGluR5, and anti-HA immunoprecipitated HA-mGluR5, but not mGluR1a. Aliquots of each cell extract (no immunoprecipitation) are in the right three lanes on each gel. ippt AB, immunoprecipitation antibody.

mGluR5 (data not shown). However, HA-mGluR5 was immunoprecipitated from extracts of cotransfected cells by either anti-HA or anti-wt antibody (Fig. 2C). Moreover, wt-mGluR5 was also immunoprecipitated from these extracts when either antibody was used (not shown). Thus, these data indicate that mGluR5 polypeptides form dimers.

To determine the specificity of dimer formation, we performed an analogous experiment in which wild type mGluR1a was cotransfected with HA-mGluR5. If the assembly of mGluR dimers is specific, mGluR1a and HA-mGluR5 should not form heterodimers. As shown in Fig. 3, antibody selective for mGluR1a did not immunoprecipitate any HA-containing bands from cells transfected with mGluR1a, mGluR1a and HA-mGluR5, or HA-mGluR5 (Fig. 3, top gel, left three lanes), but it did immunoprecipitate mGluR1 from cells transfected with mGluR1a or mGluR1a and HA-mGluR5 (Fig. 3, bottom gel, left two lanes). Similarly, the antibody selective for HA did not immunoprecipitate any mGluR1a from cells transfected with mGluR1a, mGluR1a and HA-mGluR5, or HA-mGluR5, but it did immunoprecipitate HA-mGluR5 from cells transfected with HA-mGluR5 or HA-mGluR5 and mGluR1a. Thus, despite the 60% amino acid identity between mGluR1a and mGluR5, (22), they do not heterodimerize. These data indicate that there is great specificity in the assembly of the metabotropic receptor dimers.

Metabotropic Glutamate Receptors Are Linked via Their N-terminal Extracellular Domains—To localize which part of mGluR5 was involved in dimer formation, two types of experiments were performed.

In the first set of experiments, a mutant receptor, truncated after the first transmembrane domain and tagged with the HA epitope at the C terminus, was constructed (tHA; Fig. 2A). When this mutant receptor was expressed in HEK cells and immunoprecipitated with anti-HA, it migrated during electrophoresis as a dimer (160 kDa) under nonreducing conditions and as a monomer (doublet of 80–90 kDa) under reducing conditions (Fig. 4A). Therefore, the locus for disulfide-mediated dimerization is in the N-terminal half of the receptor, most of which is extracellular.

When the tHA receptor was co-expressed with wt-mGluR5 and then immunoprecipitated with antibody to HA, an additional HA-containing band (220 kDa) was observed on the

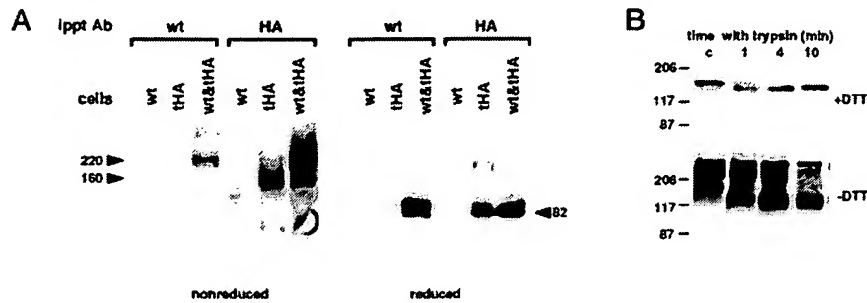


FIG. 4. The disulfide bond responsible for dimerization is in the N-terminal region of mGluR5. **A**, Western blot analysis of immunoprecipitates (ippt Ab) from SDS-solubilized membranes prepared from cells expressing wt, tHA, or both, using monoclonal anti-HA to visualize the immunoreactive bands. Antibody to the wt receptor did not immunoprecipitate any HA-positive bands from cells expressing only wt or tHA receptors but did precipitate the 220-kDa band present in co-transfected cells. Antibody to HA immunoprecipitated the 160-kDa band from tHA-expressing cells and both 220- and 160-kDa proteins from co-transfected cells. On reducing gels, all immunoprecipitated bands behaved as monomers. **B**, intact HEK cells expressing wt mGluR5 were treated with trypsin (standard 0.05% trypsin and 0.53 mM EDTA in Hank's balanced salt solution from Life Technologies, Inc.) for the times indicated, and then membranes were prepared and electrophoresed under reduced and nonreduced conditions. Trypsin treatment led to a ~17-kDa decrease in the apparent molecular mass of the monomer (reduced, top), and a loss of dimerization (nonreduced, bottom).

nonreduced gels, suggesting the formation of heterodimers between truncated-HA and wt receptors (Fig. 4A). In agreement with this interpretation, antibody to the wt receptor immunoprecipitated the 220-kDa heterodimer, but not the 160-kDa tHA homodimer, from cotransfected cells. The wt antibody did not precipitate any HA-containing species from cells transfected with only the wt or tHA mutant. Taken together, these results indicate that the truncated receptor forms both homodimers and heterodimers.

In the second experiment addressing location of the disulfide bond(s), intact HEK cells expressing the wt receptor were incubated with trypsin for various periods. We reasoned that proteolytic removal of all or part of the extracellular domain would generate a receptor fragment that does not dimerize, and from the size of this fragment we could infer the approximate location of the relevant cysteine(s). Since the wt antibody is directed toward the C terminus, all the proteolysis products observed will necessarily have intact C termini and loss of some length of the N terminus. Treatment with trypsin removed only a small fragment from the N terminus of the receptor, decreasing the apparent molecular mass by about 17 kDa (Fig. 4B, +DTT). This very limited digestion suggests that access of the protease to potential cleavage sites was restricted by steric factors due to the secondary structure of the extracellular domain. However, even very short periods of proteolysis removed the site of dimerization, since the proteolyzed receptor migrated at the monomer molecular mass under nonreducing conditions (Fig. 4B, -DTT). These results indicate that the cysteine(s) responsible for disulfide bond formation are in the N-terminal 17 kDa of mGluR5.

DISCUSSION

The experiments described here clearly show that mGluR5 normally exists as a dimer on the plasma membrane. Dimerization is mediated via a cysteine or cysteines located within 17 kDa from the N terminus in the extracellular domain. Since heterodimers between mGluR5 and mGluR1a do not form, but the truncated mGluR5 containing only the extracellular region and one transmembrane domain does dimerize, the information providing the specificity of mGluR5 dimerization also resides in the N-terminal region of the molecule. Because mGluR1a, 2-3 and 4 also migrate as dimeric species, we propose that dimerization may be a general property of the mGluR family.

Several authors who have examined mGluRs using Western blot analysis have noted the presence of high molecular mass aggregated forms of the receptors (23, 24), even in the presence

of reducing agents. In the most extreme cases these aggregates are so large that the receptor polypeptides do not enter the gel. This aggregation may reflect strong interactions among the denatured, highly hydrophobic, multiple transmembrane domains in mGluRs. In our hands, aggregation is avoided by heating samples minimally before electrophoresis (60 °C, 3 min) and by using only SDS or dodecyl maltoside as solubilizing detergents. We believe the dimer we describe is the native form of the receptor and not an artifact because: 1) it is a discrete band with a characteristic and appropriate molecular mass and not a smear, as aggregates usually are; 2) it can be converted to the monomer by reducing agents, whereas mGluR aggregates cannot; 3) even under conditions that minimize aggregation, as described above, we never observed any monomer except when samples were reduced (or proteolyzed as in Fig. 3B), suggesting that all the receptor is initially present in the membrane as dimer; and 4) the truncated mutant (tHA), which contains only a single transmembrane domain and, hence, should exhibit little tendency to aggregate, also migrated as a dimer (Fig. 3A).

To date, there is no evidence that other G protein-coupled receptors exist as covalent dimers. The α 1-adrenergic receptor of the rat ventricle behaves as a 77-kDa species in the presence or absence of DTT (25). Similarly, the electrophoretic mobility of the neuromedin B receptor is unaffected by DTT (26). A cholecystokinin receptor and an opioid receptor apparently increase in molecular mass when treated with DTT (27, 28), suggesting that disulfide bonds are maintaining compact conformations of these polypeptides. It is unlikely, therefore, that covalent, disulfide-dependent dimerization is a universal feature of G protein-coupled receptors. This structural feature may be unique to the mGluRs and perhaps the related Ca^{2+} -sensing receptors (6, 7). It is worth noting that the Ca^{2+} -sensing receptors do have the conserved cysteines characteristic of this subgroup.

The N-terminal extracellular domain of the mGluRs is related to the bacterial periplasmic binding proteins (PBPs) (12), as are extracellular domains of some iGluRs (12, 29). PBPs constitute a family of proteins involved in high affinity transport of amino acids, sugars, and other nutrients into bacteria. Three-dimensional crystal structures reported for several PBPs have indicated that these proteins are composed of two distinct globular domains with a ligand binding cleft between them (30, 31). Recently, O'Hara *et al.* (12) proposed a three-dimensional model of the structure of mGluR1a based on PBP structural information. Their alignment permitted them to

make several successful predictions concerning the glutamate binding site of the receptor, lending credence to the structural comparison. Our results indicate that the cysteine responsible for dimerization of mGluR5 is in the N-terminal 17 kDa. Within this region, there are four cysteines, all but one of which is conserved among the mGluRs. Based on the alignment of mGluRs and PBP's suggested by O'Hara *et al.* (12), it is conceivable that two of these cysteines are involved in intramolecular disulfides, leaving the remaining cysteine(s) available for intermolecular interactions.

Receptors with intracellular tyrosine kinase domains dimerize on ligand binding, and this is critical for signal transduction (32–34). Our evidence suggests that the cysteines responsible for mGluR dimerization are not those present in the cysteine-rich tyrosine kinase receptor-like domain (12). Dimerization of tyrosine kinase receptors brings the monomeric receptors into close proximity, allowing each member of the pair to phosphorylate the other, thereby providing the binding sites necessary for initiating assembly of the signal-transducing apparatus on the intracellular face of the membrane. These active tyrosine kinase receptor dimers are linked noncovalently, whereas mGluR dimers are covalently bound.

Earlier studies of adrenergic receptors emphasized the role of two disulfide-linked, conserved cysteine residues in the first and second extracellular loops. For example, in the mammalian β_2 -adrenergic receptor maintenance of this extracellular disulfide bridge is important for high affinity agonist binding and function (35, 36). In contrast, DTT had no effect on binding of ligands to muscarinic receptors (37) or prostaglandin E_2 receptors (38) but potentiated binding to and functioning of H1 histamine receptors (39–42). Therefore, one cannot predict *a priori* what effect reducing conditions will have on receptor functioning. We have preliminary results indicating that maintenance of extracellular disulfides of mGluR5 are critically important for maintenance of signal transduction through this receptor.² Consistent with this result, Vignes *et al.* (43) showed that inositol phosphate production stimulated by glutamate in synaptoneurosome was blocked by DTT, whereas that stimulated by carbachol was not. It is interesting that *N*-methyl-D-aspartate receptors are influenced oppositely by redox state: extracellular reduction leads to potentiation of receptor function, not inhibition (44–46). Perhaps ambient redox conditions lead to a complementary and coordinate regulation of iGluRs and mGluRs.

Acknowledgments—We thank U. Khosla, P. Gehlbach, S. Smout, S. Harmon, and M. Moffat for assistance at different phases of this work, Prof. S. Nakanishi for the mGluR5 and mGluR1a clones, and Peter Lukasiewicz and David Harris for comments on the manuscript.

REFERENCES

- Pin, J.-P., and Duvoisin, R. (1995) *Neuropharmacology* **34**, 1–26
- Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadowaki, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., Masu, M., and Nakanishi, S. (1993) *J. Biol. Chem.* **268**, 2836–2843
- Nomura, A., Shigemoto, R., Nakamura, Y., Okamoto, N., Mizuno, N., and Nakanishi, S. (1994) *Cell* **77**, 361–369
- Nakanishi, S. (1994) *Neuron* **13**, 1031–1037
- Eaton, S. A., Birse, E. F., Wharton, B., Sunter, D. C., Udvarhelyi, P. M., Watkins, J. C., and Salt, T. E. (1993) *Eur. J. Neurosci.* **5**, 186–189
- Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifer, O., Sun, A., Hediger, M. A., Lytton, J., and Hebert, S. C. (1993) *Nature* **366**, 575–580
- Ruat, M., Molliver, M. E., Snowman, A. M., and Snyder, S. H. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 3161–3165
- Pin, J. P., Joly, C., Heinemann, S. F., and Bockaert, J. (1994) *EMBO J.* **13**, 342–348
- Pin, J. P., Gomez, J., Joly, C., and Bockaert, J. (1995) *Biochem. Soc. Trans.* **23**, 91–96
- Gomez, J., Joly, C., Kuhn, R., Knopfel, T., Bockaert, J., and Pin, J. P. (1996) *J. Biol. Chem.* **271**, 2199–2205
- Takahashi, K., Tsuchida, K., Tanabe, Y., Masu, M., and Nakanishi, S. (1993) *J. Biol. Chem.* **268**, 19341–19345
- O'Hara, P. J., Sheppard, P. O., Thøgersen, H., Venezia, D., Haldeman, B. A., McGrane, V., Houamed, K. M., Thomsen, C., Gilbert, T. L., and Mulvihill, E. R. (1993) *Neuron* **11**, 41–52
- Tanabe, Y., Masu, M., Ishii, T., Shigemoto, R., and Nakanishi, S. (1992) *Neuron* **8**, 169–179
- Wenthold, R. J., Yokotani, N., Doi, K., and Wada, K. (1992) *J. Biol. Chem.* **267**, 501–507
- Hollmann, M., and Heinemann, S. (1994) *Annu. Rev. Neurosci.* **17**, 31–108
- Wisden, W., and Seeburg, P. H. (1993) *Curr. Opin. Neurobiol.* **3**, 291–298
- Nakanishi, S. (1992) *Science* **258**, 597–603
- Romano, C., Sesma, M. A., Macdonald, C., O'Malley, K., van den Pol, A. N., and Olney, J. W. (1995) *J. Comp. Neurol.* **355**, 455–469
- Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R., and Nakanishi, S. (1991) *Nature* **349**, 760–765
- Higuchi, R. (1990) in *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds) pp. 177–183, Academic Press, San Diego, CA
- Kolodziej, P. A., and Young, R. A. (1991) *Methods Enzymol.* **194**, 508–519
- Abe, T., Sugihara, H., Nawa, H., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1992) *J. Biol. Chem.* **267**, 13361–13368
- Baude, A., Nusser, Z., Roberts, J. D., Mulvihill, E., McIlhinney, R. A., and Somogyi, P. (1993) *Neuron* **11**, 771–787
- Petralia, R. S., Wang, Y.-X., Niedzielski, A. S., and Wenthold, R. J. (1996) *Neuroscience* **71**, 949–976
- Terman, B. I., and Insel, P. A. (1986) *J. Biol. Chem.* **261**, 5603–5609
- Kusui, T., Benya, R. V., Battey, J. F., and Jensen, R. T. (1994) *Biochemistry* **33**, 12968–12980
- Bone, E. A., and Rosenzweig, S. A. (1988) *Peptides* **9**, 373–381
- Gioannini, T. L., Liu, Y. F., Park, Y. H., Hiller, J. M., and Simon, E. J. (1989) *J. Mol. Recog.* **2**, 44–48
- Stern-Bach, Y., Bettler, B., Hartley, M., Sheppard, P. O., O'Hara, P. J., and Heinemann, S. F. (1994) *Neuron* **13**, 1345–1357
- Quiocho, F. A. (1990) *Phil. Trans. R. Soc. Lond.* **326**, 341–351
- Adams, M. D., and Oxender, D. L. (1989) *J. Biol. Chem.* **264**, 15739–15742
- Heldin, C. H., Ernlund, A., Rorsman, C., and Ronnstrand, L. (1989) *J. Biol. Chem.* **264**, 8905–8912
- Qian, X., LeVea, C. M., Freeman, J. K., Dougall, W. C., and Greene, M. I. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 1500–1504
- Earp, H. S., Dawson, T. L., Li, X., and Yu, H. (1995) *Breast Cancer Res. Treat.* **35**, 115–132
- Dohlman, H. G., Caron, M. G., DeBlasi, A., Frielle, T., and Lefkowitz, R. J. (1990) *Biochemistry* **29**, 2335–2342
- Noda, K., Saad, Y., Graham, R. M., and Karnik, S. S. (1994) *J. Biol. Chem.* **269**, 6743–6752
- Wei, J. W., and Sulakhe, P. V. (1980) *Naunyn Schmiedeberg's Arch. Pharmacol.* **314**, 51–59
- Michalak, M., Wandler, E. L., Strynadka, K., Catena, R., Liu, H. J., and Olley, P. M. (1992) *Biochim. Biophys. Acta* **1111**, 247–255
- Carman-Krzan, M. (1984) *Agents Actions* **14**, 561–565
- Dickenson, J. M., and Hill, S. J. (1994) *Biochem. Pharmacol.* **48**, 1721–1728
- Donaldson, J., and Hill, S. J. (1986) *Br. J. Pharmacol.* **87**, 191–199
- Donaldson, J., and Hill, S. J. (1987) *Br. J. Pharmacol.* **90**, 263–271
- Vignes, M., Guirmand, J., Sassetti, I., and Recasens, M. (1992) *Neurochem. Int.* **21**, 229–235
- Tang, L. H., and Aizenman, E. (1993) *J. Physiol. (Lond.)* **465**, 303–323
- Reynolds, I. J., Rush, E. A., and Aizenman, E. (1990) *Br. J. Pharmacol.* **101**, 178–182
- Sucher, N. J., Wong, L. A., and Lipton, S. A. (1990) *Neuroreport* **1**, 29–32

² C. Romano, I. Saito, and K. L. O'Malley, unpublished observation.

Covalent and Noncovalent Interactions Mediate Metabotropic Glutamate Receptor mGlu₅ Dimerization

CARMELO ROMANO, JUDITH KELVIN MILLER, KRZYSZTOF HYRC, SETA DIKRANIAN, STEVEN MENNERICK, YUTAKA TAKEUCHI, MARK P. GOLDBERG, and KAREN L. O'MALLEY

Departments of Ophthalmology and Visual Sciences (C.R., S.D.), Anatomy and Neurobiology (C.R., J.K.M., K.L.O.), Neurology (K.H., M.P.G.), and Psychiatry (S.M.), Washington University School of Medicine, St. Louis, Missouri; and Banyu Tsukuba Research Institute, Tsukuba, Japan (Y.T.)

Received March 29, 2000; accepted September 25, 2000

This paper is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Some, perhaps all, G protein-coupled receptors form homo- or heterodimers. We have shown that metabotropic glutamate receptors are covalent dimers, held together by one or more disulfide bonds near the N terminus. Here we report how mutating cysteines in this region affect dimerization and function. Covalent dimerization is preserved when cysteines 57, 93, or 99 are mutated but lost with replacement at 129. Coimmunoprecipitation under nondenaturing conditions indicates that the C[129]S mutant receptor remains a dimer, via noncovalent interactions. Both C[93]S and C[129]S bind [³H]quisqualate, whereas binding to C[57]S or C[99]S mutants is absent or

greatly attenuated. The C[93]S and C[129]S receptors have activity similar to wild-type when assayed by fura-2 imaging of intracellular calcium in human embryonic kidney cells or electrophysiologically in *Xenopus laevis* oocytes. In contrast, C[57]S or C[99]S are less active in both assays but do respond with higher glutamate concentrations in the oocyte assay. These results demonstrate that 1) covalent dimerization is not critical for mGlu₅ binding or function; 2) mGlu₅ remains a noncovalent dimer even in the absence of covalent dimerization; and 3) high-affinity binding requires Cys-57 and Cys-99.

Many classes of receptors, such as the tyrosine kinase-linked receptors and the ligand-gated ion channels, function as di- or oligomeric assemblies of polypeptides. In contrast, G protein-coupled receptors (GPCRs) have traditionally been thought to exist and operate as monomers. This view has been changing. For example, early indirect evidence, from radiation inactivation target-size analysis (Venter and Fraser, 1983) and gel electrophoresis of receptors (Brett and Findlay, 1979; Avissar et al., 1983), suggested that GPCRs are part of larger multimeric structures. More recently, direct functional and structural studies indicated that at least some GPCRs are multimers, most often homodimers. For instance, chimeric receptors composed of an N-terminal sequence from a muscarinic receptor and C-terminal half from an α -adrenergic receptor neither bind ligand nor activate effector systems, but these functions are restored by coexpression with a chimera composed of an adrenergic N-terminal half and a cholinergic C-terminal half (Maggio et al., 1993). Similarly, expression of the metabotropic γ -aminobutyric acid (mGABA₁ or GABA-B1) receptor yields a polypep-

tide that binds radiolabeled antagonist but does not bind agonist or couple to G protein-coupled inwardly rectifying potassium channels unless the mGABA₂ receptor polypeptide is coexpressed (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Ng et al., 1999). Such complementation experiments indicate that receptor functioning requires interactions between more than one receptor polypeptide. Studies demonstrating that δ - and κ -opioid receptors individually form homodimers and also heterodimerize to form a hybrid with unique properties (Cvejic and Devi, 1997; Jordan and Devi, 1999) further substantiate a dimer/oligomer model.

Diverse structural mechanisms underlie GPCR dimerization. For example, catecholamine receptor dimers are held together by noncovalent protein-protein interactions involving the transmembrane domains (Hebert et al., 1996; Ng et al., 1996), whereas the mGABA (Kuner et al., 1999) and δ -opioid receptors (Cvejic and Devi, 1997) dimerize via noncovalent interactions mapped to regions in the intracellular C-terminal region. We have shown that dimers of mGluRs are covalently linked by disulfide bonds located extracellularly, within ≈ 17 kDa of the N terminus (Romano et al., 1996). Subsequently, the Ca²⁺-sensing receptors (CaRs),

This work was supported by National Institutes of Health Grants MH57817 and EY02687, an unrestricted grant from Research to Prevent Blindness, and the McDonnell Center for Cellular and Molecular Neuroscience.

ABBREVIATIONS: GPCR, G protein-coupled receptor; mGluR, metabotropic glutamate receptor; HA, hemagglutinin epitope; HEK, human embryonic kidney; AM, acetoxymethyl ester; t, truncated; h, human; wt, wild-type; DTT, dithiothreitol; mGABA, metabotropic γ -aminobutyric acid; CaR, Ca²⁺-sensing receptor; PBP, periplasmic binding protein.

which are homologous to the mGluRs and share many conserved cysteines, have also been shown to dimerize by this mechanism (Bai et al., 1998). Finally, there is evidence that muscarinic cholinergic receptors may covalently dimerize via extracellular cysteines located in the o2 and o3 loops (Zeng and Wess, 1999).

In this study, we examined the structural basis and functional role of dimerization of mGlu₅ using a set of mutant receptors. We demonstrate that Cys-129 is critically important for covalent dimerization but conclude that this interaction is not necessary for agonist binding or receptor function. We propose that Cys-57 and Cys-99 participate in an intramolecular disulfide bond not critical for covalent dimerization, but important for the integrity of the ligand binding site. We also demonstrate that novel noncovalent interactions, probably involving the extracellular domains, participate in mGluR dimerization.

Materials and Methods

Construction of Mutant Receptors. All of the point mutations introduced into the mGlu₅ coding sequence were generated by recombinant polymerase chain reaction (Higuchi, 1990) using sense and antisense primers containing the relevant cysteine to serine mutation. Primers included: 5'-AGAGGAAGTCTGCTGCAG-3', C[57]S; 5'-CACTTGGCTCTGAGATCA-3', C[93]S; 5'-AGATTCCTCTGGCATTG-3', C[99]S; 5'-TGGTACGCTCTGTAGATG-3', C[129]S; and their complements. The C[57,93,99,129]S mutant as well as all other multiple mutations were generated by using one of the single mutation constructs as a template together with an additional mutant primer pair. The resulting double mutant then served as a template for further mutagenesis etc. The truncated wild-type or hemagglutinin (HA)-tagged mGlu₅ construct was described previously (Romano et al., 1996). The truncated mutant constructs were created by digesting their respective full-length clones with *Bst*II and *Not*I then ligating them with a truncated wild-type fragment encoding the first transmembrane region and C-terminal domain cut with the same enzymes. All of the mutant full-length and truncated clones were confirmed by sequencing.

Cell Culture and Transfections. HEK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Grand Island, NY) and incubated at 37°C with 5% CO₂. For experiments requiring Western blotting and/or immunoprecipitation, plasmid DNA was transfected into subconfluent HEK cells with either LT1 (PanVera, Madison, WI) or Eugene6 (Roche Diagnostics, Nutley, NJ) according to the manufacturer's instructions, using a ratio of 3 µl of transfecting reagent per 1 µg of DNA. Cells were harvested 24 h after transfection.

For ratiometric Ca²⁺ imaging, DNA was introduced into HEK cells via electroporation, by a modification of published protocols (Puchalski and Fahl, 1992; Gomez et al., 1996; Ishmael et al., 1996). DNA (8 µg) was electroporated into 3 to 4 × 10⁶ HEK cells in 300 µl of Ca²⁺-, Mg²⁺-free PBS with a Bio-Rad Gene Pulser (0.4-cm electrode gap cuvette, 2 pulses, 25 µF, 600 ohms, 1 kV). Cells were subsequently kept at room temperature for 5 min, diluted in Dulbecco's modified Eagle's medium enriched with 10% fetal calf serum, and plated on poly-D-lysine-treated dishes with 35-mm grids (Mat-Tek, Ashland, MA). Cells were allowed to recover for 15 h before imaging.

Membranes, SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. HEK cells transfected with plasmids encoding wild-type (wt) or mutant mGlu₅ were washed twice with PBS, then incubated for 10 min at room temperature in PBS containing 12 mM *N*-ethylmaleimide. This buffer was replaced by ice-cold lysis buffer composed of 2 mM HEPES, 2 mM EDTA, plus protease inhibitors (Complete tablets, Boehringer Mannheim, Germany). Cells were scraped and then disrupted in a tight-fitting, motor-driven, Teflon

and glass homogenizer. Nuclei were pelleted (1000g, 5 min) and discarded. Membranes were pelleted by centrifugation (35,000g, 40 min) and usually used without further washes. Membrane proteins were separated by electrophoresis in 7.5% Laemmli gels. The sample buffer for reducing gels contained 20 mM dithiothreitol (DTT) or 5% 2-mercaptoethanol. Samples were incubated at 60°C for 3 min before loading; samples were never boiled. Western blotting was done as described previously (Romano et al., 1996), using polyvinylidene difluoride membranes (Immobilon; Millipore Corp., Milford, MA) and chemiluminescent visualization. Antibody against mGlu₅ was an affinity-purified polyclonal raised against the C-terminal 13 amino acids, as described previously (Romano et al., 1996). Antibodies against the HA epitope were from BABCO (Berkeley, CA).

Immunoprecipitations. For immunoprecipitations, membranes were homogenized in immunoprecipitation buffer (40 mM HEPES, 400 mM NaCl, protease inhibitors as above, pH 7.5) containing either 0.5% SDS (at 60°C for complete, denaturing solubilization) or 0.5% dodecyl maltoside (at room temperature, for gentle, nondenaturing solubilization). The SDS extract was diluted 5-fold into immunoprecipitation buffer containing 0.5% dodecyl maltoside (to sequester free SDS into mixed micelles, thereby permitting immunoprecipitation) and protease inhibitors. The solubilized extract was then centrifuged at 100,000g for 35 min to remove any undissolved material, anti-receptor antibody was added, and the solution was incubated at 4°C overnight. Protein A (or protein G)-Sephrose (Sigma, St. Louis, MO) was added, and the incubation was continued for 2 h at room temperature on a rocking table. The protein A pellets were washed three times with PBS before elution with sample buffer and electrophoresis.

[³H]Quisqualate Binding Assay. To increase the yield of receptors and thereby maximize signal, total cell membranes were used for the [³H]quisqualate binding assay. Cells were washed twice in PBS (without *N*-ethyl maleimide, which decreased binding), lysed in hypotonic lysis buffer as above, and centrifuged at 17,000g for 35 min. The pellet was resuspended in buffer containing 40 mM HEPES, 2.5 mM Ca²⁺, and protease inhibitors. Usually, 600 nM [³H]quisqualate was present in a final assay volume of 100 µl. Incubation was for 60 min at 25°C, and bound label was separated from free label by fast filtration over #30 filters (Schleicher & Schuell, Keene, NH). Nonspecific binding was determined in the presence of 1 mM glutamate.

Single Cell Fluorescent Ratiometric Measurements of Intracellular Ca²⁺. Cytosolic calcium determination upon stimulation of cells with glutamate or carbachol was performed using the fluorescent calcium indicator fura-2 (Grynkiewicz et al., 1985) as described previously (Hyrz et al., 1997). The fura-2 was bath-loaded at 37°C by incubation for 30 min with 6 µM fura-2/AM (Molecular Probes, Eugene, OR) and 0.12% of Pluronic F-127, followed by another 30-min incubation at 37°C to allow for hydrolysis of the AM ester. Calcium measurements were carried out using standard ratio-imaging techniques. Cells loaded with fura-2 were imaged on an inverted microscope (Nikon Diaphot, Nikon Inc., Melville, NY) using a 40×, 1.3 numerical aperture fluorite oil immersion objective (Nikon) and an ICCD camera (Hamamatsu Photonics, Oak Brook, IL). A 75-W xenon arc lamp was used to provide fluorescence excitation. The excitation wavelengths were selected by using band-specific filters (340HT15 and 380HT15; Omega Optical, Brattleboro, VT) in combination with an XF73 dichroic mirror (Omega Optical).

Ratio images were obtained by acquiring pairs of images at alternate excitation wavelengths (340/380 nm) and filtering the emission at >510 nm (EFLP filter, Omega Optical). Image acquisition and processing were controlled by a personal computer connected to the camera and filter wheel (MetaFluor, Universal Imaging Corp., West Chester, PA). Image pairs were captured every 10 s and digitized, and the images at individual wavelengths were averaged over four frames. All imaging experiments were carried out at room temperature in a HEPES- and bicarbonate-buffered salt solution containing 116 mM NaCl, 5.4 mM KCl, 1 mM NaH₂PO₄, 25 mM NaHCO₃, 1.8

mM CaCl₂, 1 mM MgSO₄, 12 mM HEPES, and 5.5 mM D-glucose, pH 7.45 ± 0.2.

Immunocytochemistry. After image analysis, cells were analyzed immunocytochemically to confirm receptor expression. Cells were washed with PBS for 5 min, fixed for 15 min at room temperature with 4% paraformaldehyde in PBS, washed with PBS for 5 min, and further processed or stored in 5 ml of PBS at 4°C until analyzed for receptor content the next day. The cells were washed two more times for 5 min per wash; blocked for 30 min with 1% bovine serum albumin, 0.1% Triton, in PBS; incubated 1 h at 37°C in 1% bovine serum albumin, 0.1% Triton in PBS containing the polyclonal rabbit anti-mGluR5; washed three times for 5 min per wash with PBS; incubated in the dark for 45 min at room temperature with goat-anti-rabbit Cy3 (Jackson ImmunoResearch, West Grove, PA) in 1% bovine serum albumin, 0.1% Triton in PBS; washed three times for 5 min per wash with PBS; stored in PBS; and observed and photographed under fluorescence at magnification 200. In the case of N-terminally HA-tagged receptors (see Fig. 5C), surface expression was confirmed by performing the labeling in the absence of detergent, using anti-HA (Covance, Richmond, CA) as primary antibody.

Activation of Ca²⁺-Dependent Cl⁻ Channels in *Xenopus laevis* Oocytes. Linearized pcDNA3 plasmids containing wild-type or mutant mGlu₅ coding sequences were transcribed using an in vitro transcription kit (mMESSAGE mMACHINE, Ambion, Austin, TX). Transcript levels and integrity were verified on formaldehyde RNA gels. Oocytes were harvested from *X. laevis* under tricaine (0.1%) anesthesia. Oocytes were enzymatically defolliculated in a collagenase (2 mg/ml) saline solution containing 96 mM NaCl, 2.5 mM KCl, 1.0 mM MgCl₂, and 10 mM HEPES, pH 7.4 at 37°C for 20 min. Oocytes were maintained at 18°C in the above saline solution with 1.8 mM CaCl₂ and supplemented with theophylline (0.5 mM) and sodium pyruvate (0.55 mg/ml). Individual oocytes were injected with 40 ng of wild-type or mutant complementary RNA and were assayed electrophysiologically 2 to 8 days after injection.

Oocyte electrophysiology was performed using an Axoclamp 2-B amplifier (Axon Instruments, Foster City, CA) in the two-electrode voltage-clamp configuration. Recording pipettes filled with 3 M KCl had an open tip resistance of approximately 1 MΩ. For recording, individual oocytes were washed for 15 to 30 min with calcium-containing saline solution lacking the theophylline and pyruvate supplements. Oocytes were placed in a 100-μl chamber and were constantly perfused (2 ml/min) during the experiment with unsupplemented saline solution.

Endogenous calcium-dependent chloride currents, activated by 20-s applications of 100 μM glutamate, were used to detect mGluR activation. Oocytes were clamped at -20 mV, near the chloride equilibrium potential to avoid intracellular chloride shifts during mGluR activation. The membrane potential was briefly (20 ms) pulsed to +30 mV once per second. The outward chloride current at the end of this 20-ms voltage pulse was measured before, during, and after perfusion of glutamate.

Results

Mutating Cysteine 129 Disrupts Covalent Dimerization of mGlu₅. Previous results indicated that the disulfide bond(s) responsible for covalent dimerization of mGlu₅ involve(s) a cysteine or cysteines located within ≈17 kDa of the N terminus (Romano et al., 1996). There are four cysteines in this region, residues 57, 93, 99, and 129. Three of these, 57, 99, and 129, occupy positions conserved across the entire family of mGluRs and CaRs.

To determine whether these cysteines were involved in disulfide-dependent dimer formation, we adopted the strategy of mutating to serine and examining the consequences of these manipulations for receptor structure. Under nonreducing conditions, receptors mutagenized at Cys-129 migrated

at the monomeric molecular weight, whereas wt, C57S, C93S, and C99S migrated as dimers (Fig. 1). These data indicate that Cys-129 participates in interactions critical for covalent dimerization.

Previous results indicated that a truncated form of mGlu₅ (composed of the extracellular N terminus, one transmembrane domain, and the first nine amino acids of intracellular loop 1 in-frame with the final 32 amino acids of the C terminus) was also a covalent dimer, and, when coexpressed, would assemble into heterodimers with the full-length receptor (Romano et al., 1996). When an epitope-tagged version of this truncated receptor (tm5HA) was coexpressed with each of the set of full-length receptors mutated at individual cysteines, heterodimers formed between tm5HA and the wt, C57S, C93S mutants, but not with C99S or C129S (Fig. 2).

We then examined the behavior of single and multiple cysteine mutants of the truncated mGlu₅. When electrophoresed under nonreducing conditions, truncated mGlu₅ with wild-type cysteines migrated as a dimer, but the tetra-mutated tC[53,93,99,129]S migrated as a monomer (Fig. 3, wt, t[57,93,99,129]). This is consistent with the hypothesis that one or more of these cysteines participate in the disulfide bond necessary for covalent dimerization. When tCys-57, tCys-93, or tCys-99 were individually mutated to serine, a small fraction of each of these truncated receptors migrated as monomer under nonreducing conditions, but the majority appeared to be intact dimer. The tC[129]S mutant, on the other hand, migrated nearly exclusively as a monomer (Fig. 3). Interestingly, for all the mutants examined, there was often a fraction of the receptor polypeptide that migrated at a molecular weight greater than that of the dimer, although this was variable from experiment to experiment. This suggests that assembly of mutant truncated receptors may be imperfect. When Cys-129 was left intact, but Cys-57, Cys-93, and Cys-99 were altered to serines, covalent dimerization was also lost. In fact, when any combination of three of the first four cysteines was altered, dimerization was lost (Fig. 3). Collectively, these data strongly support the contention that covalent dimerization of mGlu₅ critically depends on Cys-129.

Noncovalent Dimerization of Truncated mGlu₅ Is Present in tC[129]. The absence of disulfide-mediated covalent dimerization does not preclude the possibility that mutant receptor polypeptides may exist as dimers held together by noncovalent protein-protein interactions. To test for noncovalent interactions dependent on the N-terminal region of the receptor, coimmunoprecipitation experiments were performed using the truncated receptors under conditions that would either preserve or disrupt noncovalent interactions. Cells were

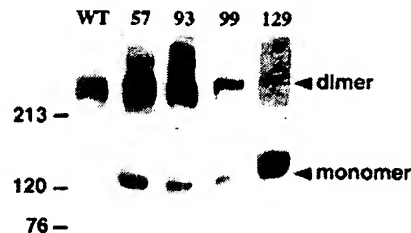


Fig. 1. Western blot analysis of wild-type and cysteine-mutated mGlu₅ expressed in HEK cells. Samples were prepared without reduction. The numbers above the gel lanes signify the position of the cysteine that has been changed to serine.

cotransfected with the HA-tagged truncated wild-type mGlu₅, together with truncated receptors containing the wild-type C-terminal epitope but mutated at selected cysteines. Membranes were prepared from these cells, then solubilized under gentle (room temperature, buffer containing 0.5% dodecyl maltoside) or denaturing (0.5% SDS, 3 min heating at 60°C) conditions. An aliquot of each membrane preparation was used to confirm receptor expression by Western blotting (Fig. 4, top two panels). The remainder of each extract was immunoprecipitated with antibody against the wild-type C terminus, and the presence of HA-tagged mutant receptor in the immunoprecipitate was determined by Western blotting. Despite denaturation, the HA tagged-truncated receptor was coimmunoprecipitated when it was expressed in the presence of truncated wild-type receptor, as expected for a covalent heterodimer (Fig. 4C). Similar results were observed with C[57]S, C[93]S, and C[99]S (data not shown) as expected, because these receptors also appear to be covalent dimers (Fig. 3). Mutation of the first four cysteines, or of Cys-129 individually, led to the loss of coimmunoprecipitation under the denaturing conditions (Fig. 4C). This is consistent

with the results presented in Figs. 1 through 3 demonstrating the absence of covalent dimerization in these mutants. In contrast, when gentler, nondenaturing conditions were used to solubilize the membranes, HA-tagged receptor was present in the immunoprecipitate when either t-wt or tC[129]S, but not tC[57,93,99,129]S, were coexpressed (Fig. 4D). These data indicate that the tC[129]S mutant exists as a noncovalent dimer with the receptor containing wild-type cysteines. This noncovalent dimerization is not present when all of the first four cysteines have been mutated.

Effect of Cysteine Mutations on [³H]Quisqualate Binding to mGlu₅. To determine whether elimination of covalent dimerization in mGlu₅ alters the properties of the receptor binding site, binding studies using [³H]quisqualate as a radioligand were performed. Relative to the full-length wt receptor, the C[57,93,99,129]S mutant had greatly reduced detectable binding of [³H]quisqualate (Table 1). When the individual cysteines were mutated, binding was preserved with C[93]S and C[129]S but effectively eliminated with C[57]S or C[99]S. Similar results were obtained when analogous mutations of the truncated receptor were analyzed (Table 1). Receptor expression was confirmed by Western blotting. Because covalent dimerization is lost in the tC[129]S mutants but binding is preserved, agonist binding is not critically dependent on covalent dimerization.

Accurate values for dissociation constants are very difficult to obtain by saturation analysis using a relatively low affin-

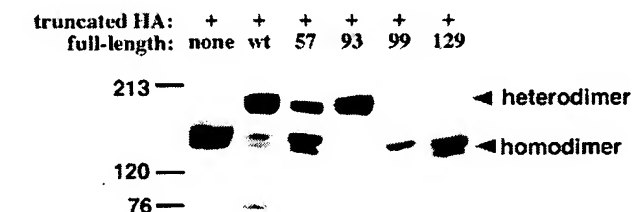


Fig. 2. Western blot analysis of HA-tagged, truncated mGlu₅ coexpressed with wild-type and cysteine-mutated full-length mGlu₅ in HEK cells. Samples were prepared without reduction. The numbers above the gel are as in Fig. 1. A monoclonal antibody to the HA-epitope was used for visualization of the HA-tagged truncated mGlu₅.

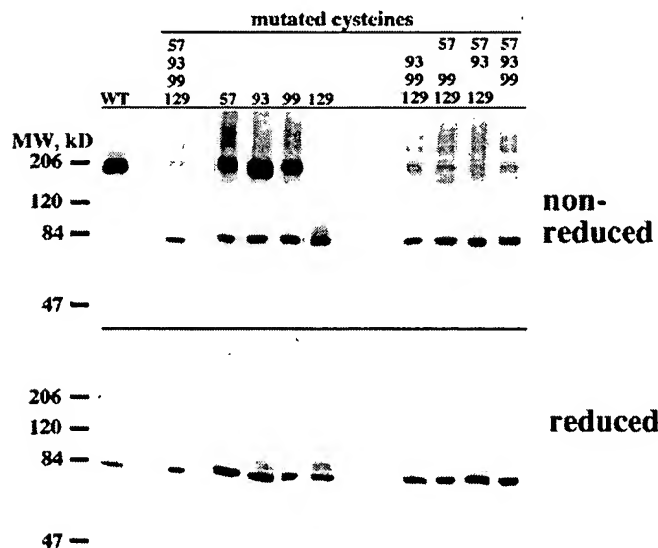


Fig. 3. Western blot analysis of wild-type and cysteine-mutated truncated mGlu₅ expressed in HEK cells. For the top gel, samples were prepared without reduction; for the bottom gel, samples were reduced using 20 mM DTT. Each lane represents a different mutant. The numbers above the gel lanes signify the position of the cysteine or cysteines that have been changed to serines. Under reduced conditions, all receptors migrated as ~80 kDa monomers. Under nonreduced conditions, the wt, C[57]S, C[93]S, and C[99]S mutants migrate mostly as dimers, whereas the C[129]S and the multiply mutated receptors, migrate primarily as monomers.

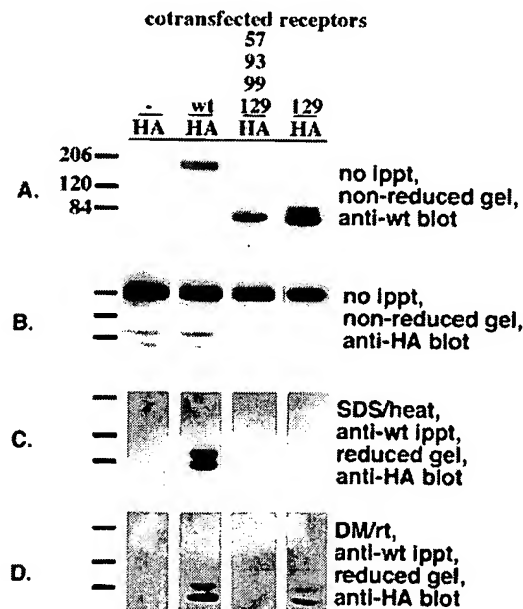


Fig. 4. Coimmunoprecipitation of the epitope-tagged receptor together with the cysteine-mutated receptors demonstrates covalent and noncovalent dimerization. HEK cells were transfected with truncated mGlu₅ containing the HA epitope at the C terminus (-/HA) or cotransfected with this receptor and a truncated mGlu₅ containing the wt C terminus and wt or mutated cysteines as indicated. The top panel demonstrates expression of the receptors bearing the wt C terminus and the cysteine alterations. The second panel demonstrates expression of HA-tagged receptor in each cotransfected cell type. The third panel demonstrates that, upon solubilization under denaturing conditions (0.5% SDS, 60°C), only receptors containing all wt cysteines will coprecipitate the HA-tagged receptor. The fourth panel illustrates that, with gentle solubilization (0.5% dodecyl maltoside, room temperature), the C[129]S mutant will also coprecipitate the HA-tagged receptor but the multiple mutant will not.

ity radioligand such as [³H]quisqualate. Nonetheless, saturation analysis was performed with full-length versions of the wild-type and C[129]S mutant receptors. Wild-type receptor bound [³H]quisqualate with a K_D value of 514 nM (± 187 nM, $N = 3 \pm$ S.E.M.), whereas the C[129]S mutant bound with a K_D value of 583 nM (± 57 nM, $N = 3 \pm$ S.E.M.). Thus, the binding affinities for [³H]quisqualate are similar between the wild-type and C[129]S mutant, although subtle differences may exist that will be more readily assessed when higher affinity radioligands are available.

Effect of Cysteine Mutations on mGlu₅-Induced Calcium Mobilization in HEK Cells. Glutamate-induced mobilization of intracellular calcium was analyzed by fura-2 fluorescence microscopy in HEK cells transiently transfected with wt or mutant full-length mGlu₅. Representative responses of two individual cells transfected with the indicated mutant are shown in the upper part of Fig. 5; a compilation of the peak responses from all cells are shown below. All the results shown reflect the responses of cells that both responded to carbachol (which activates an endogenous muscarinic receptor on HEK cells) and had confirmed expression of transfected mGlu₅ (via post hoc fixation, immunocytochemistry, and field relocation).

Cells transfected with a noncoding vector never showed a calcium response to added glutamate. In contrast, almost all cells expressing wild-type mGlu₅ responded to glutamate with an increase in intracellular calcium (Fig. 5). In most cells, the response was oscillatory, and the oscillations persisted for many minutes after washout of glutamate. Similar responses were seen with C[93]S (not shown) and with the dimerization-defective mutant C[129]S (Fig. 5). Many of the cells transfected with the C[57]S mutant had a calcium response, but persistent oscillations were rare. A much smaller fraction of cells transfected with the other mutants responded, but never strongly, or with persistent oscillations. Taken together, these results demonstrate that covalent dimerization is not essential for receptor functioning but alterations in several individual or multiple cysteines disrupt receptor-mediated calcium responses.

Although we only counted responses from individual cells that had been confirmed immunocytochemically to express the wild-type or mutant receptors, another experiment was performed to better document that the poorly responding single mutants were reaching the cell surface. An HA-epitope tag was added to the N terminus, immediately after the presumed signal sequence, of receptors with all wt cysteines

or the C[57]S or C[99]S mutations. Cells were incubated with anti-HA followed by fluorescent secondary antibody without permeabilization and visualized. Similar numbers of cells were labeled with a similar intensity (Fig. 5C). Therefore, mutation of Cys-57 or Cys-99 does not prevent receptor transport and expression on the plasma membrane. The possibility of misfolding leading to altered surface expression or function of the triple mutants has not yet been explored.

Effect of Cysteine Mutations on mGlu₅ Activation of Endogenous Ca²⁺-Dependent Cl⁻ Channels in *X. laevis* Oocytes. When expressed in *X. laevis* oocytes, wild-type Group I mGluRs robustly activate the endogenous Ca²⁺-dependent Cl⁻ channels. Responses of mGlu₅ wild-type and mutant receptors to the addition of 100 μ M glutamate were similar to those seen with the calcium-imaging studies (Fig. 6, A and B). Specifically, mutation of nonconserved Cys-93, or covalent dimerization-disrupting Cys-129, maintained an activity level comparable with wild-type, whereas C[57]S, C[99]S, or C[57,93,99,129] gave no, or greatly attenuated, responses. Interestingly, C[57]S and C[99]S respond similarly to wild-type when exposed to 20 mM glutamate (Fig. 6C).

Discussion

The structural bases and functional consequences of GPCR dimerization are just beginning to be explored. Previously, we demonstrated that mGluRs covalently dimerize via extracellular disulfide bonds. The present findings demonstrate that covalent dimerization of mGlu₅, mediated by disulfide bonds, is not critical for the functioning of this receptor. This conclusion is based on the identification of a mutant that differs from wild-type by a single amino acid, C[129]S, in which covalent dimerization is absent, yet the receptor is synthesized, exported to the plasma membrane, binds agonist, and serves to mobilize intracellular calcium in a manner indistinguishable from wild-type. The presence of noncovalent dimerization was also identified. Thus, covalent and noncovalent bonds participate in mGluR dimerization.

A model of mGluR structure was previously proposed by O'Hara et al. (1993). These investigators identified sequence and secondary structure homologies between the ionotropic and metabotropic glutamate receptors and a structurally well defined class of bacterial proteins known as the periplasmic binding proteins (PBPs). Based on these similarities, as well as receptor mutagenesis, they proposed a bi-lobed "Venus Flytrap" model for the extracellular ligand-binding domain of mGluRs. Further confirmation of this model, in particular the extracellular location of the binding site in mGluRs, was found in studies showing that binding is preserved when the truncated extracellular domains of mGlu₁ or mGlu₄ were expressed as secreted, soluble proteins (Okamoto et al., 1998; Han and Hampson, 1999). Additionally, recent chimeric and point mutation studies of the closely related CaR (Brauner-Osborne et al., 1999) and the more distantly related mGABA receptor (Malitschek et al., 1999) demonstrated that the extracellular domain of these receptors also could fold and bind agonists in a manner analogous to the mGluRs. Finally, molecular modeling studies of the mGABA₁ receptor suggest an even closer fit to the PBP-like structure than exhibited by mGluRs or CaRs (Galvez et al., 1999).

TABLE 1
Binding of [³H]quisqualate to mGlu₅ and mutants in HEK cell membranes

	Binding Relative to wt \pm S.D. ($n = 4$)
fl, wt^a	1.00 (= 910 fmol/mg protein)
fl, C(57)S	0.00 \pm 0.01
fl, C(93)S	1.51 \pm 0.25
fl, C(99)S	0.00 \pm 0.01
fl, C(129)S	1.23 \pm 0.20
$\text{fl, C(57, 93, 99, 129)S}$	0.11 \pm 0.15
t-wt	1.00 (= 948 fmol/mg protein)
tC(57)S	0.00 \pm 0.01
tC(93)S	1.75 \pm 0.05
tC(99)S	0.03 \pm 0.03
tC(129)S	2.25 \pm 0.63
$\text{tC(57, 93, 99, 129)S}$	0.18 \pm 0.14

^a fl = full-length; t = truncated.

Our previous work suggesting that the disulfide bond responsible for dimerization was located within 20 kDa of the N terminus, together with the model inferred from the PBP crystallography data (O'Hara et al., 1993). This permitted us to predict a role for Cys-129 in dimerization. Specifically, of the four possible cysteines in this region, Cys-93 is not conserved, and Cys-57 and Cys-99 probably form an intramolecular disulfide bond as described below. Only Cys-129 would be located on the outer edge of the "Venus Flytrap" in a region removed from the core of the binding pocket and not predicted to be near other free cysteines. Inasmuch as the mutant C[129]S failed to covalently dimerize, the proposed model received further confirmation.

High resolution crystallographic studies of at least six different PBPs have established the presence of an intramolec-

ular disulfide bond ensuring that one side of the ligand-binding pocket is covalently held in place (Sack et al., 1989). An analogous pair of cysteines has been conserved in the mGABA receptor, and mutation of these residues leads to the loss of binding. However, DTT treatment of membranes containing wild-type mGABA receptor did not prevent binding, leading Galvez et al. (1999) to suggest that this disulfide bond is important for the correct initial folding, but not maintenance, of the active conformation of the binding site. Interestingly, mGluRs and CaRs, which are less homologous to the mGABA receptors or PBPs and more similar to each other, have not conserved one of the pivotal cysteines involved in the PBP-like intramolecular disulfide. Rather, mGluRs and CaRs have a conserved cysteine located in the insert between the region homologous to the first β -sheet and

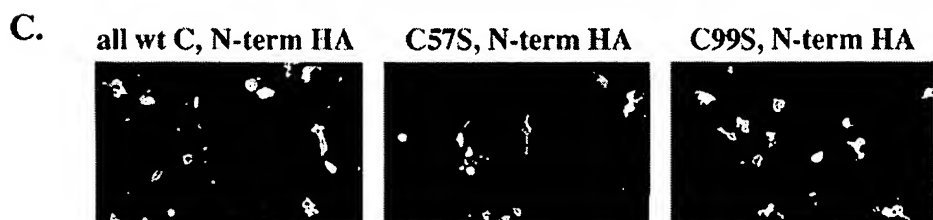
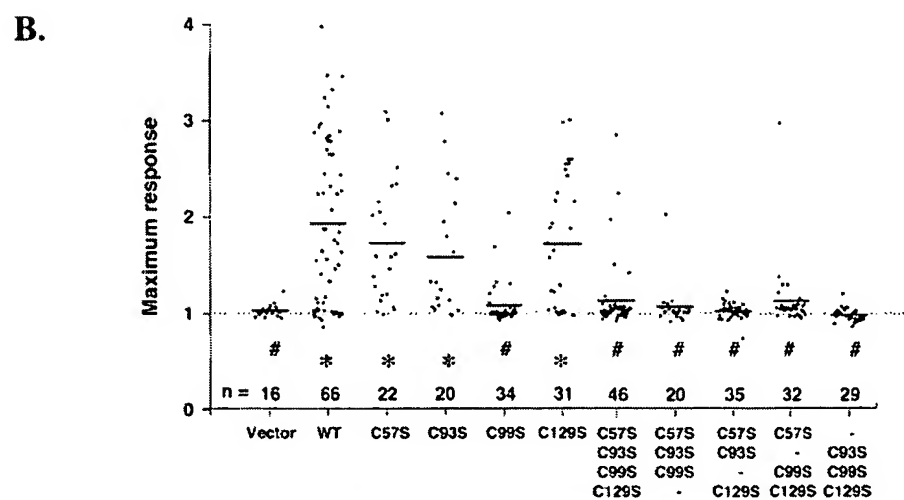
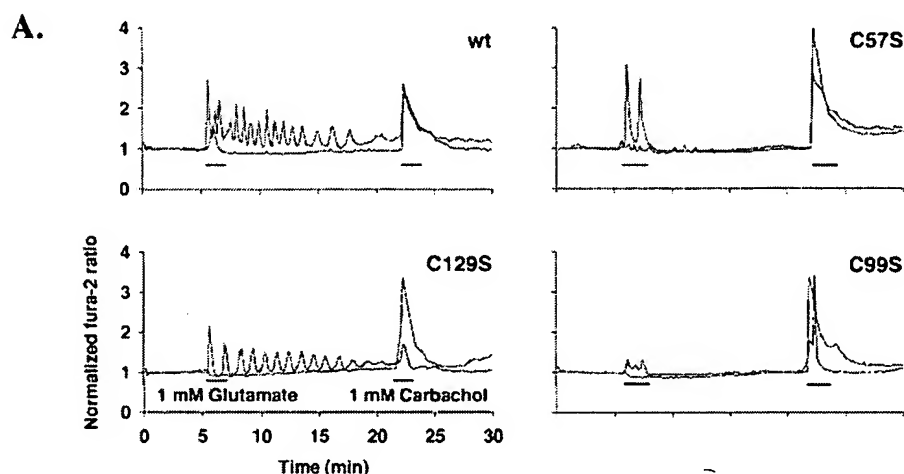


Fig. 5. Changes in intracellular free $[Ca^{2+}]$ induced by glutamate in HEK cells expressing full-length wt and cysteine-mutated mGlu₆. **A**, representative traces from two cells for each of six different receptors. The first bar under each of the traces indicates the time of exposure to 1 mM glutamate; the second bar indicates the time of exposure to 1 mM carbachol. **B**, compiled data from all cells. Each data point represents the maximum response to glutamate in a single cell, and the horizontal bars show the average responses of all cells for each mutant. The number of cells of each type is indicated. The statistical significance of the differences ($\alpha = 0.05$) was assessed using one-way ANOVA on ranks. *, different from those of nontransfected controls; #, different from wild-type. The cells included in the analysis were pooled from 4 to 13 independent experiments (2 for vector controls). **C**, immunocytochemical labeling under nonpermeabilizing conditions using an anti-HA-antibody demonstrates surface expression of N-terminally HA-tagged mGlu₆ (wild-type, C[57]S, or C[99]S).

the first α -helical region of the PBP [see (Brauner-Osborne et al. (1999) and Galvez et al. (1999) for alignments]. The alignment of O'Hara et al. (1993) of the secondary structure elements would place this cysteine (Cys-57 of mGlu₅) in close proximity to the cysteine that is completely conserved throughout the known PBPs, mGABAs, CaRs, and mGluRs (Cys-99 of mGlu₅). Therefore, given the spatial proximity predicted by the molecular modeling studies (O'Hara et al.,

1993; Brauner-Osborne et al., 1999; Galvez et al., 1999), it seems reasonable to propose that Cys-57 and Cys-99 of mGlu₅ form an intramolecular disulfide bond that would be in approximately the same position as the disulfide bond of the PBPs. Mutation of these residues would be expected to disrupt function. Indeed, negligible binding of [³H]quisqualate to either C[57]S or C[99]S (or any of the triple mutations, including these residues, data not shown) was observed (Table 1). Moreover, there was no functional response to 100 μ M glutamate when either of these mutants were expressed in *X. laevis* oocytes but the C[57]S mutant did exhibit a transient response to glutamate in the HEK cell Ca²⁺ mobilization assay. Both C[57]S and C[99]S responded in *X. laevis* oocytes when tested at 20 mM glutamate. Presumably, this reflects an extant, but greatly decreased affinity of agonists for the C[57]S and C[99]S mutant receptors. Taken together, these results are consistent with important roles for Cys-57 and Cys-99 in the structure of the agonist binding site.

Covalent, disulfide-dependent dimerization has also been reported in a human CaR (Bai et al., 1998), although there are conflicting reports concerning the effects of mutating cysteines on receptor dimerization and function. In one study (Pace et al., 1999), mutating the residue equivalent to mGlu₅ Cys-129 (hCaR Cys-131) did not disrupt dimerization, whereas individual mutations at positions homologous to Cys-99 and Cys-240 (hCaR Cys-101 and Cys-236) partially disrupted dimerization, and mutating both residues prevented dimerization. In contrast, Ray et al. (1999) noted that there are two cysteines in this region (hCaR Cys-129 and Cys-131), and covalent dimerization of the receptor is prevented when both are mutated to serine. Pace et al. (1999) did not examine the properties of this double mutant. Interestingly, introduction of a new cysteine between the mutagenized sites restored covalent dimerization (Ray et al., 1999). Thus, either Cys-129 or Cys-131 (or both) of the hCaRs participate in disulfide-dependent dimerization. Functionally, this dimerization-deficient double mutant is properly glycosylated, is expressed on the cell surface, and actually has a higher affinity for Ca²⁺ than the wild-type receptor (31). These results are quite analogous to the findings presented here.

Our results indicate that mGluRs dimerize via noncovalent bonds as well as disulfide bonds, although neither the mechanism nor the functional role of this noncovalent dimerization has been determined. We have identified some mutants in which noncovalent as well as covalent dimerization has been disrupted (data not shown), however, poor expression or misfolding make straightforward interpretation of such results difficult at present. Because the tetramutated tC[57,93,99,129]S lost the ability to coimmunoprecipitate under nondenaturing conditions, this may indicate that the extracellular domain contains moieties critical to noncovalent dimerization. We cannot rule out, however, the possibility that the single remaining transmembrane portion of this truncated receptor affects this process. Additional experiments will be required to address this model. Because each functional mutant we have studied is either a covalent or noncovalent dimer, dimerization may be required for functioning. This has not been adequately tested however. Localization of the specific residues involved in noncovalent dimerization should help elucidate the role that the dimerized state plays in the functioning of the mGluRs.

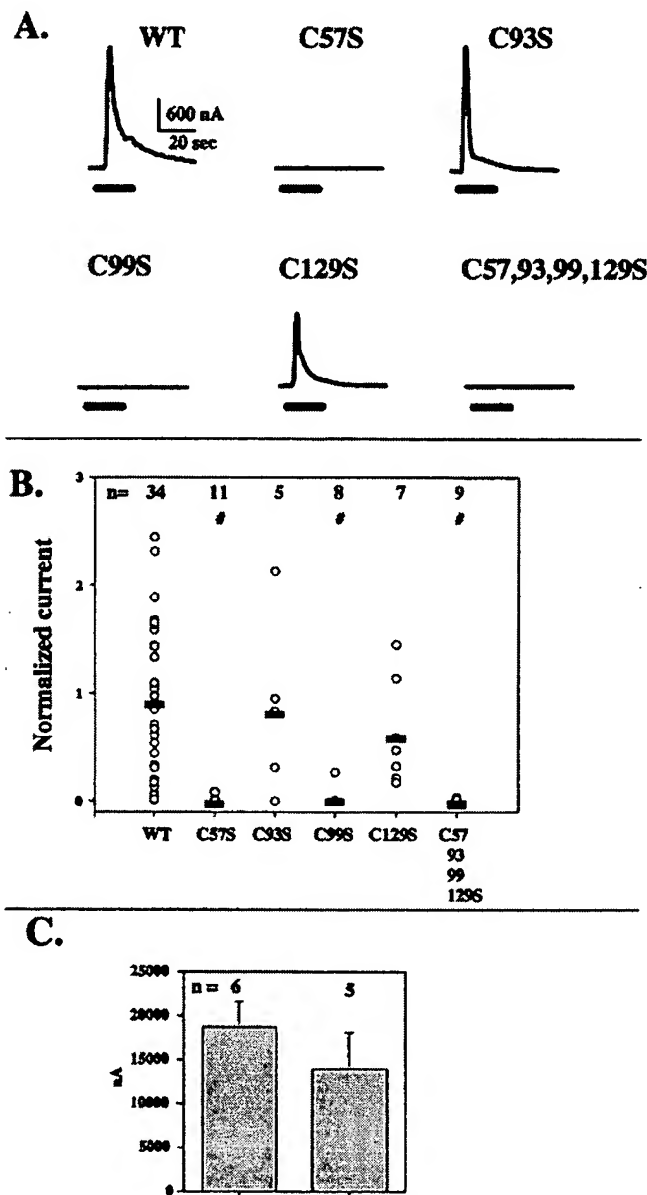


Fig. 6. Calcium-dependent chloride currents induced by glutamate in *X. laevis* oocytes expressing full-length wt and cysteine-mutated mGlu₅. **A**, representative traces for each of five different receptors. The bar under each trace indicates the time of exposure to 100 μ M glutamate. Note that wt, C[93]S, and C[129]S responded, whereas the others did not. **B**, compiled data from all oocytes tested at 100 μ M glutamate. Each data point represents the maximum response to glutamate in a single oocyte, and the horizontal bars show the average response of all cells for each mutant. The number of cells tested is indicated. The statistical significance of the differences ($\alpha = 0.05$) was assessed using one-way ANOVA on ranks. #, different from wt. C, responses to 20 mM glutamate by oocytes injected with C[57]S or C[99]S.

Several different types of protein-protein interactions have been described as responsible for the noncovalent bonds between GPCR polypeptides. For example, D2 and β -adrenergic receptor dimers appear to involve interactions between transmembrane domains (Hebert et al., 1996; Ng et al., 1996), whereas dimerization of mGABA receptors involves a region of the intracellular C-terminal domain (Kuner et al., 1999). Our experiments demonstrate that a site of noncovalent interaction between mGlu₅ monomers is likely to be in the extracellular domain. Dimerization of the bradykinin B2 receptor has recently been shown to involve the extracellular N terminus (Abdalla et al., 1999).

Given the presence of noncovalent dimers of mGlu₅, and the activity of the C[129]S mutant, it is not clear what the precise function of disulfide-mediated covalent dimerization of mGluRs may be. Because it is not required for agonist binding or signal transduction measured in the standard ways, it must serve a more elusive role. Mechanistically, covalent dimerization might "lock-in" noncovalent interactions, thereby contributing to dimer specificity and/or stability. An additional speculation is that covalent dimerization is related to modulation of signal transduction, perhaps involving receptor desensitization or the kinetics of ligand binding or second-messenger formation. Alternatively, covalent dimerization may be important for a function not directly related to signal transduction, perhaps involving interaction with an extracellular target on the plasma membrane of the pre- or postsynaptic neuron, a glial cell, or the extracellular matrix.

References

- Abdalla S, Zaki E, Lother H and Quitterer U (1999) Involvement of the amino terminus of the B₂ receptor in agonist-induced receptor dimerization. *J Biol Chem* 274:26079–26084.
- Avisar S, Amitai G and Sokolovsky M (1983) Oligomeric structure of muscarinic receptors is shown by photoaffinity labeling: Subunit assembly may explain high- and low-affinity agonist states. *Proc Natl Acad Sci USA* 80:156–159.
- Bai M, Trivedi S and Brown EM (1998) Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. *J Biol Chem* 273:23605–23610.
- Brauner-Osborne H, Jensen AA, Sheppard PO, O'Hara P and Krosgaard-Larsen P (1999) The agonist-binding domain of the calcium-sensing receptor is located at the amino-terminal domain. *J Biol Chem* 274:18382–18386.
- Brett M and Findlay JB (1979) Investigation of the organization of rhodopsin in the sheep photoreceptor membrane by using cross-linking reagents. *Biochem J* 177: 215–223.
- Cvejic S and Devi LA (1997) Dimerization of the delta opioid receptor: Implication for a role in receptor internalization. *J Biol Chem* 272:26959–26964.
- Galvez T, Parmentier ML, Joly C, Malitschek B, Kaupmann K, Kuhn R, Bittiger H, Froestl W, Bettler B and Pin JP (1999) Mutagenesis and modeling of the GABA_B receptor extracellular domain support a Venus flytrap mechanism for ligand binding. *J Biol Chem* 274:13362–13369.
- Gomez J, Mary S, Brabet I, Parmentier ML, Restituito S, Bockaert J and Pin JP (1996) Coupling of metabotropic glutamate receptors 2 and 4 to G α 15, G α 16, and chimeric G α q/i proteins: Characterization of new antagonists. *Mol Pharmacol* 50:923–930.
- Gryniewicz G, Poenie M and Tsien RY (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450.
- Han G and Hampson DR (1999) Ligand binding to the amino-terminal domain of the mGluR4 subtype of metabotropic glutamate receptor. *J Biol Chem* 274:10008–10013.
- Hebert TE, Moffett S, Morello JP, Loisel TP, Bichet DG, Barret C and Bouvier M (1996) A peptide derived from a beta₂-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J Biol Chem* 271:16384–16392.
- Higuchi R (1990) Recombinant PCR, in *PCR Protocols* (1990) A Guide to Methods and Applications (Innis MA, Gelfand DH, Sninsky JJ and White TJ eds) pp 177–183, Academic Press, San Diego.
- Hyre K, Handran SD, Rothman SM and Goldberg MP (1997) Ionized intracellular calcium concentration predicts excitotoxic neuronal death: Observations with low-affinity fluorescent calcium indicators. *J Neurosci* 17:6669–6677.
- Ishmael JE, Franklin PH, Murray TF and Leid M (1996) High level expression of the NMDAR1 glutamate receptor subunit in electroporated COS cells. *J Neurochem* 67:1500–1510.
- Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA and Gerald C (1998) GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. *Nature (Lond)* 396:674–679.
- Jordan BA and Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature (Lond)* 399:697–700.
- Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A and Bettler B (1998) GABA(B) receptor subtypes assemble into functional heteromeric complexes. *Nature (Lond)* 396:683–687.
- Kuner R, Kohr G, Grunewald S, Eisenhardt G, Bach A and Kornau HC (1999) Role of heteromer formation in GABA(B) receptor function. *Science (Wash DC)* 283:74–77.
- Maggio R, Vogel Z and Wess J (1993) Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein-linked receptors. *Proc Natl Acad Sci USA* 90:3103–3107.
- Malitschek B, Schweizer C, Keir M, Heid J, Froestl W, Mosbacher J, Kuhn R, Henley J, Joly C, Pin JP, Kaupmann K and Bettler B (1999) The N-terminal domain of γ -aminobutyric acid_B receptors is sufficient to specify agonist and antagonist binding. *Mol Pharmacol* 56:448–454.
- Ng GY, Clark J, Coulombe N, Ethier N, Hebert TE, Sullivan R, Kargman S, Chateaufort A, Tsukamoto N, McDonald T, Whiting P, Mezey E, Johnson MP, Liu Q, Kolakowski LF Jr, Evans JF, Bonner TI and O'Neill GP (1999) Identification of a GABA_B receptor subunit, gb2, required for functional GABA_B receptor activity. *J Biol Chem* 274:7607–7610.
- Ng GY, O'Dowd BF, Lee SF, Chung HT, Brann MR, Seeman P and George SR (1996) Dopamine D2 receptor dimers and receptor-blocking peptides. *Biochem Biophys Res Commun* 227:200–204.
- O'Hara PJ, Sheppard PO, Thihetagersen H, Venezia D, Haldeman BA, McGrane V, Houamed KM, Thomsen C, Gilbert TL and Mulvihill ER (1993) The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron* 11:41–52.
- Okamoto T, Sekiyama N, Otsu M, Shimada Y, Sato A, Nakanishi S and Jingami H (1998) Expression and purification of the extracellular ligand binding region of metabotropic glutamate receptor subtype 1. *J Biol Chem* 273:13089–13096.
- Pace AJ, Gama L and Breitwieser GE (1999) Dimerization of the calcium-sensing receptor occurs within the extracellular domain and is eliminated by Cys → Ser mutations at Cys¹⁰¹ and Cys²³⁶. *J Biol Chem* 274:11629–11634.
- Puchalski RB and Fahl WE (1992) Gene transfer by electroporation, lipofection, and DEAE-dextran transfection: Compatibility with cell-sorting by flow cytometry. *Cytometry* 13:23–30.
- Ray K, Hauschild BC, Steinbach PJ, Goldsmith PK, Hauache O and Spiegel AM (1999) Identification of the cysteine residues in the amino-terminal extracellular domain of the human Ca²⁺ receptor critical for dimerization: Implications for function of monomeric Ca²⁺ receptor. *J Biol Chem* 274:27642–27650.
- Romano C, Yang WL and O'Malley KL (1996) Metabotropic glutamate receptor 5 is a disulfide-linked dimer. *J Biol Chem* 271:28612–28610.
- Sack JS, Saper MA and Quiocho FA (1989) Periplasmic binding protein structure and function: Refined X-ray structures of the leucine/isoleucine/valine-binding protein and its complex with leucine. *J Mol Biol* 206:171–191.
- Venter JC and Fraser CM (1983) The structure of alpha- and beta-adrenergic receptors. *Trends Pharmacol Sci* 4:256–258.
- White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Ford SM and Marshall FH (1998) Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature (Lond)* 396:679–682.
- Zeng FY and Wess J (1999) Identification and molecular characterization of m3 muscarinic receptor dimers. *J Biol Chem* 274:19487–19497.

Send reprint requests to: Carmelo Romano, Ph.D., Department of Ophthalmology & Visual Sciences, Campus Box 8096, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110. E-mail: romano@vision.wustl.edu email

Novel allosteric antagonists shed light on mglu₅ receptors and CNS disorders

Will P.J.M. Spooren, Fabrizio Gasparini, Thomas E. Salt and Rainer Kuhn

Although multiple metabotropic glutamate (mglu) receptor subtypes were cloned in the early 1990s, progress in the characterization of these receptors has been slow because of difficulties in obtaining subtype-selective ligands. However, in the past few years exciting progress has been made on the mglu₅ receptor subtype following the identification of selective non-amino-acid-like ligands that implicate the mglu₅ receptor as a potentially important therapeutic target, particularly for the treatment of pain and anxiety.

To date, eight subtypes of metabotropic glutamate (mglu) receptors have been cloned and classified into three groups on the basis of sequence similarities, and pharmacological and biochemical properties: Group I mglu receptors (mglu₁ and mglu₃), Group II mglu receptors (mglu₂ and mglu₄) and Group III mglu receptors (mglu₆, mglu₇, and mglu₈)¹.

Although Group I mglu receptors are highly related, both mglu₁ and mglu₃ receptors have a distinct expression pattern in the brain, which clearly suggests differential roles in nervous

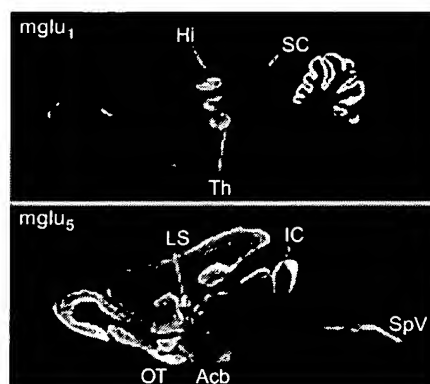


Fig. 1. Metabotropic glutamate Group I (mglu₁ and mglu₃) receptor-like immunoreactivity in rat brain parasagittal sections. Abbreviations: Acb, nucleus accumbens; Hi, hippocampus; IC, inferior colliculus; LS, lateral septal nucleus; OT, olfactory tubercle; SC, superior colliculus; SpV, spinal trigeminal nuclei; Th, thalamus. Reproduced, with permission, from Ref. 3.

system function (Table 1)^{2,3}. mglu₅ receptors are found most abundantly throughout the cerebral cortex, hippocampus, basal ganglia and some parts of the spinal cord (Fig. 1). Recently, subtype-selective ligands for the mglu₅ receptor were discovered and their effects in animal models of various nervous system disorders characterized.

Non-amino-acid-like mglu₅ receptor antagonists

A large number of mglu receptor ligands have been described and can be classified into two groups according to their mode of inhibition⁴. The vast majority of compounds are amino acid derivatives that interact competitively at the glutamate-binding site located in the large extracellular N-terminal domain. Particularly for Group I receptors, selective competitive antagonists are very scarce, have low potency and do not show subtype selectivity. The most potent compound is currently LY393675 (Fig. 2), a nonselective antagonist with IC₅₀ values of 0.48 and 0.35 μ M at mglu₅ and mglu₁ receptors, respectively⁴. More recently, LY344545 (Fig. 2) has been described as a selective competitive antagonist at the mglu₅

receptor with a selectivity factor of seven for mglu₅ over mglu₁ receptors. However, LY344545 has low potency (IC₅₀ = 5.5 μ M) and also antagonizes Group II receptors in a similar potency range⁵. Despite these significant advances, potent and subtype-selective competitive ligands have not been identified. Moreover, amino-acid-derived ligands generally show poor blood-brain barrier penetration, thus limiting their use.

Recently, novel mglu receptor antagonists were identified using functional assays by screening compound libraries for non-amino-acid-like substances. The first subtype-selective mglu₅ receptor antagonists reported were SIB1757 and SIB1893 (Fig. 2) with IC₅₀ values of 3.7 and 3.5 μ M, respectively, in the inositol phosphate (IP) accumulation assay⁶. Shortly after, 2-methyl-6-(phenylethynyl)-pyridine (MPEP) was described⁷, a 100-fold more potent antagonist derived from chemical variation of SIB1893. At human mglu_{5a} receptors, expressed in recombinant cells, and rat mglu₅ receptors in neonatal cortical slices, MPEP inhibited quisqualate-stimulated IP production with IC₅₀ values of 36 nM and 17.9 nM, respectively, but had no significant agonist or antagonist activities at cells expressing other metabotropic or ionotropic glutamate receptor subtypes at concentrations up to 10 μ M (Ref. 7). However, at high concentrations (i.e. 20 μ M and 200 μ M, respectively) 1000–10 000-fold above its IC₅₀, MPEP inhibited NMDA receptor activity by 22.6% and 64.9% in electrophysiological recordings of rat cultured primary cortical neurones⁸. Details of the mechanism and site of action of these new antagonists are provided in Box 1. Furthermore, extracellular recordings in the hippocampus showed that responses to iontophoretic application of the selective Group I mglu receptor agonist (*R,S*)-2,5-dihydroxyphenylglycine (DHPG) but not AMPA were inhibited by MPEP (either iontophoretically or intravenously)⁷; similarly, iontophoretic application of

Table 1. Distribution of mglu₁ and mglu₅ receptors in the rat brain^{a,b}

Rat brain region	mglu ₁	mglu ₅
Cortex	+	+++(+)
Caudate-putamen	+	+++
Nucleus accumbens	+	+++
Olfactory tubercle	+	+++
Globus pallidus	++	++
Substantia nigra	+	+
Subthalamic nucleus	++	+
Hippocampus		
CA1	+	+++
CA3	+	+++
dentate gyrus	++	+++
Cerebellum (Purkinje cell layer)	+++	-
Spinal cord		
lamina I–III	+	+++
lamina IV–V	+	+

^aAbbreviation: mglu, metabotropic glutamate.

^bSymbols: -, no detectable expression; +, low expression; ++, moderate expression; +++, high expression. Adapted and modified from Refs 2 and 3.

MPEP reduced neuronal responses in the thalamus to the selective mglu₅ receptor agonist (*R,S*)-2-chloro-5-hydroxyphenylglycine (CHPG) compared with those responses to NMDA (Ref. 11), indicating that MPEP is also a selective mglu₅ receptor antagonist *in vivo* (Box 2).

Physiology of mglu₅ receptors

Activation of Group I receptors can elicit a variety of postsynaptic effects on central neurones such as depolarization via a reduction of K⁺ conductance or an increase in inward cation current¹. This typically causes increases in neural firing and potentiation of synaptic inputs. Presynaptic effects of Group I receptor activation have also been demonstrated¹. The development of selective mglu₁ and mglu₅ receptor antagonists now enables the investigation of some of these actions mediated by mglu₁ and/or mglu₅ receptors specifically. Interestingly, in the subthalamic nucleus *in vitro*, the depolarizing effect via reduction of K⁺ conductance of Group I receptor activation is attributable solely to mglu₅ receptors, even though both mglu₁ and mglu₅ receptors are present¹⁰. The converse appears to be the case in the thalamus *in vitro*, where similar effects appear to be mediated specifically via mglu₁ receptors even though mglu₅ receptors are known to be present⁹. This indicates very specific membrane effects that are dependent on the

brain region, which might be very important both functionally and pharmacologically. It is noteworthy that such mglu receptor activation by exogenous agonists *in vitro* is not necessarily accompanied by obvious excitatory synaptic events¹⁰, possibly reflecting that conditions in slices are not optimal to reveal physiological release of glutamate onto such receptors^{9,10}. In this respect, it is interesting that in the thalamus *in vivo*, activation of either mglu₁ or mglu₅ receptors can have an excitatory effect, and that both receptors appear to possess synaptic roles¹¹.

Specific modulation of NMDA responses by mglu₅ receptor activation occurs in several brain areas^{11–13}. Together with the probable specific colocalization of receptors in synaptic and peri-synaptic areas, this offers a highly selective method of mglu₅ receptor potentiation of NMDA-receptor-mediated synaptic activity. However, *in vivo* (where neuronal membranes are not voltage clamped) there could also be a modulation of synaptic responses as a result of the effects of mglu₅ receptor activation on membrane resistance and potential, and this will be less selective^{11,14,15}. Thus, there might be at least two ways in which mglu₅ and/or mglu₁ receptors could potentiate synaptic responses, and it might be that these have a different functional relevance, perhaps in terms of time-course or in synaptic plasticity¹⁶. Interestingly, mglu₁-

receptor-mediated potentiation of ionotropic responses might involve protein kinase C, whereas the apparently similar effect of mglu₅ receptors might not¹⁵. The newly available antagonists should play a significant role as tools in further in-depth investigations.

Box 1. Allosteric mglu receptor antagonists

Metabotropic glutamate (mglu) receptors are members of the family III of G-protein-coupled receptors (GPCRs), which is characterized by a large N-terminal extracellular region and includes the Ca²⁺-sensing receptor, GABA_B receptors and some vomeronasal receptors. Homology modelling to bacterial periplasmic binding proteins^a, chimeric receptor analysis^{b,c}, and X-ray crystallography of a soluble extracellular region of the mglu₁ receptor in complex with glutamate^d have shown that the extracellular region is responsible for ligand binding. The mglu receptor family therefore differs from the classical rhodopsin-type GPCRs, where the ligand-binding site occurs within the seven transmembrane (7TM) domain.

The discovery of novel mglu receptor antagonists^{e–g} that are structurally unrelated to amino acids triggered the hypothesis that such compounds might act at novel receptor sites through allosteric mechanisms. Indeed, a detailed study by Pagano *et al.*^h showed that 2-methyl-6-(phenylethynyl)-pyridine (MPEP) is a noncompetitive antagonist

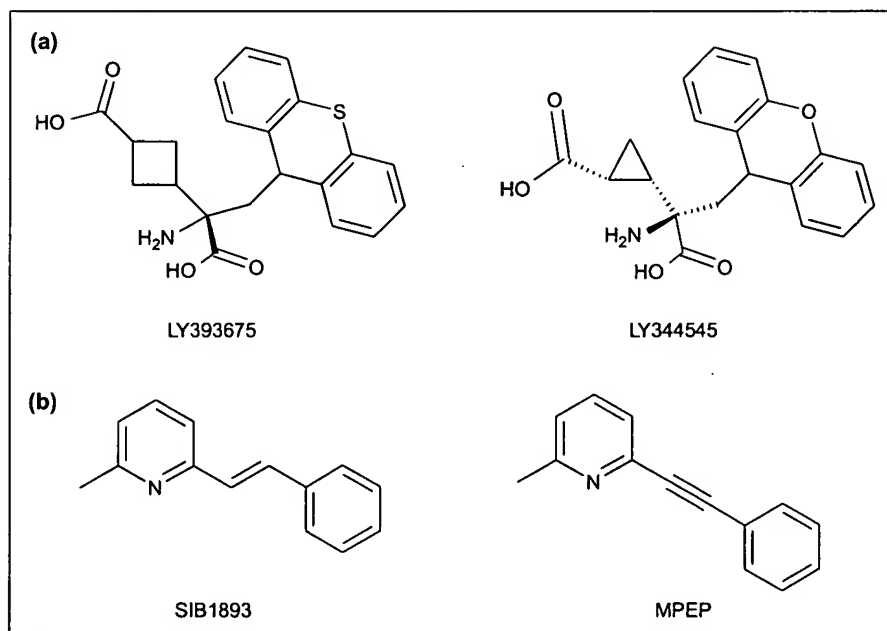


Fig. 2. Structures of Group I metabotropic glutamate (mglu) receptor antagonists. (a) LY393675 is a nonselective mglu₁ and mglu₅ receptor antagonist whereas LY344545 is a selective mglu₅ receptor antagonist. (b) SIB1893 and MPEP [2-methyl-6-(phenylethynyl)-pyridine] are recently developed noncompetitive and selective mglu₅ receptor antagonists.

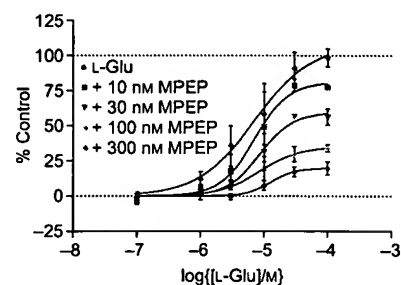


Fig. 1. Noncompetitive inhibition of glutamate (L-Glu)-stimulated inositol phosphate (IP) production with increasing concentrations of 2-methyl-6-(phenylethynyl)-pyridine (MPEP) in human metabotropic glutamate type 5a (mglu_{5a})-receptor-expressing cells. Data are expressed as percentage of IP production over basal level and are mean \pm SEM of three experiments performed in triplicates. Reproduced, with permission, from Ref. h.

In vivo effects of mglu₅ receptor antagonists

The available selective and brain-penetrable mglu₅ receptor antagonists (see above) have been applied in various nervous system disease models.

Pain and analgesia

Pharmacological and *in vivo* electrophysiological studies suggest a role for Group I mglu receptors in nociceptive processes^{1,17}, and the possible role(s) of mglu₅ receptors are highlighted in Box 2 (see also Table 2).

Anxiety and depression

Given the expression of mglu₅ receptors in limbic forebrain regions, a role in psychiatric conditions such as anxiety and depression was hypothesized and tested in validated animal models. In the so-called conditioned response tests

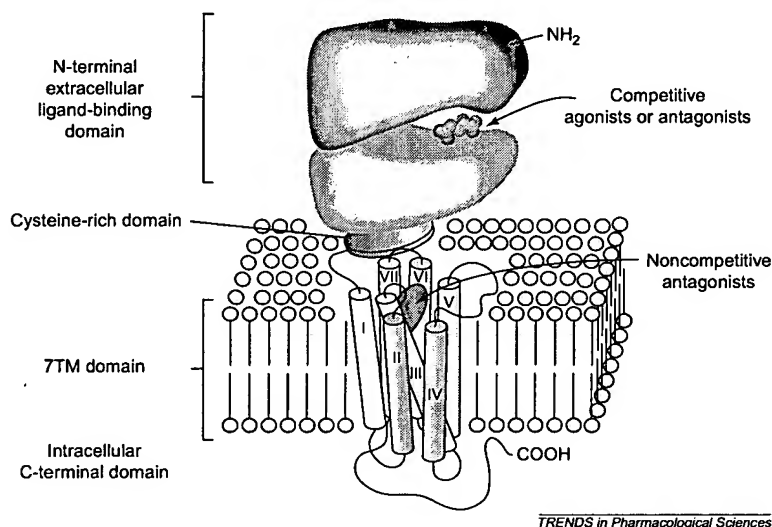


Fig. II. A monomeric form of the metabotropic glutamate type 5 (mglu₅) receptor, depicting the binding site of noncompetitive antagonists in the seven-transmembrane (7TM) domain and of competitive agonists or antagonists in the large N-terminal extracellular domain.

(Fig. I) that inhibits mglu₅ receptor function without affecting binding of glutamate to the extracellular region. MPEP also inhibited to a large extent constitutive receptor activity in cells overexpressing mglu₅ receptors, which suggests that MPEP acts as an inverse agonist.

Consistent with these functional data, high-affinity binding of [³H]M-MPEP (a tritiated analogue of MPEP) to chimeric receptors and point mutants of mglu₅ receptors specifically required the amino acid residues Pro655 and Ser658 in TMIII and Ala810 in TMVII. Recently described non-amino-acid-like antagonists that are structurally unrelated to MPEP and are selective for mglu₁ receptors¹⁴ or mglu₁ and mglu₅ receptors^k share this mechanism and site of action in the 7TM domain.

Several important implications can be derived from these findings.

(1) Novel regulators of family III GPCRs can act as competitive ligands at the extracellular agonist-binding site or as allosteric ligands in the 7TM domain (Fig. II). (2) The amino acid sequence of the allosteric binding site in the 7TM domain seems to be less conserved and to tolerate large structural variations, which will greatly facilitate the discovery of subtype-selective antagonists for other family III GPCRs. (3) It is not known how noncompetitive antagonists inhibit receptor activity but it can be assumed that activation of family III GPCRs requires a series of conformational changes in the extracellular ligand binding domain that are transmitted to the 7TM domain. Binding of noncompetitive antagonists in the 7TM domain can specifically affect conformational changes of the 7TM domain and thus have no direct effect on the occupation of the agonist binding site.

References

- a O'Hara, P.J. *et al.* (1993) The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron* 11, 41–52
- b Takahashi, K. *et al.* (1993) Role of the large extracellular domain of metabotropic glutamate receptors in agonist selectivity determination. *J. Biol. Chem.* 268, 19341–19345
- c Tones, M.A. *et al.* (1995) The agonist selectivity of a class III metabotropic glutamate receptor, human mGluR4a, is determined by the N-terminal extracellular domain. *NeuroReport* 7, 117–120
- d Kunishima, N. *et al.* (2000) Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* 407, 971–977
- e Annoura, H. *et al.* (1996) A novel class of antagonists for metabotropic glutamate receptors, 7-(hydroxyimino)-cyclopropa[b]chromen-1a-carboxylates. *Bioorg. Med. Chem. Lett.* 6, 763–766
- f Varney, M.A. *et al.* (1999) SIB-1757 and SIB-1893: selective, noncompetitive antagonists of metabotropic glutamate receptor type 5. *J. Pharmacol. Exp. Ther.* 290, 170–181
- g Gasparini, F. *et al.* (1999) 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu₅ receptor antagonist. *Neuropharmacology* 38, 1493–1503
- h Pagano, A. *et al.* (2000) The noncompetitive antagonists 2-methyl-6-(phenylethynyl)pyridine and 7-hydroxyiminocyclopropan [b]chromen-1a-carboxylic acid ethyl ester interact with overlapping binding pockets in the transmembrane region of group I metabotropic glutamate receptors. *J. Biol. Chem.* 275, 33750–33758
- i Litschig, S. *et al.* (1999) CPCCOEt, a noncompetitive metabotropic glutamate receptor 1 antagonist, inhibits receptor signaling without affecting glutamate binding. *Mol. Pharmacol.* 55, 453–461
- j Carroll, F.Y. *et al.* (2000) Pharmacological characterization of BAY36-7620, a novel antagonist selective for mGluR1 receptors. *Soc. Neurosci.* 26, 618.4, 1649
- k van Wagenen, B.C. *et al.* (2000) Structure–activity relationship studies of NPS2390: a potent and selective group I metabotropic glutamate receptor antagonist. *Soc. Neurosci.* 26, 618.3, 1649

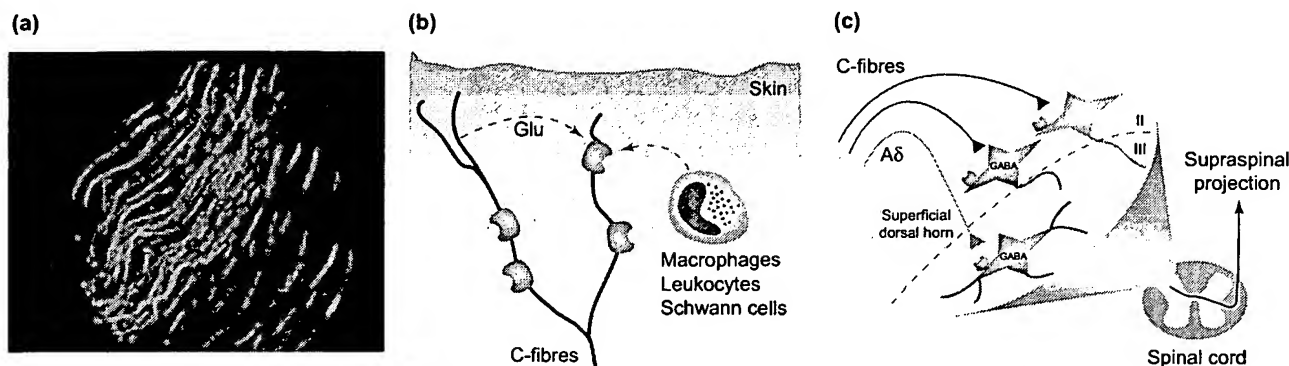
Box 2. Role of mglu_5 receptors in pain and analgesia

Fig. 1. (a) Immunohistochemical staining of a rat skin section with a rabbit polyclonal antibody recognizing the metabotropic glutamate type 5 (mglu_5) receptor. Green fluorescence represents neuronal tubulin, and red fluorescence represents mglu_5 receptor staining, respectively. Reproduced, with permission, from Ref. 8. (b) Possible activation of mglu_5 receptors on C-fibres by glutamate released from afferents or other cells in the skin following, for example, injury or inflammation. (c) Activation of dorsal horn GABA-containing and non-GABA-containing (possibly excitatory) neurones by C and A δ afferents terminating in laminae II and III (Ref. c). mglu_5 receptors are represented by blue elements. Note that other glutamate receptors have been omitted for clarity.

Metabotropic glutamate type 5 (mglu_5) receptor protein and/or mRNA have been localized at several levels of the somatosensory neuraxis from the peripheral endings of nociceptive C-fibre afferents^a (Fig. 1a), dorsal root ganglia^b and superficial dorsal horn neurones^{b-e} to the thalamus and cerebral cortex^{f,g}. This suggests an intimate involvement of mglu_5 receptors in nociceptive processes. Recent studies using 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and SIB1757 show that blockade of peripheral mglu_5 receptors can reverse inflammation-induced mechanical

hyperalgesia^{a,h} and neuropathy-induced thermal hyperalgesiaⁱ. By contrast, neuropathy-induced tactile allodynia and mechanical hyperalgesia appear unaffected^{h,i}. This indicates that peripheral mglu_5 receptors are activated in certain hyperalgesic states, presumably by glutamate released from either primary afferents or other non-neural cells^{j-l} (Fig. 1b). Given the peripheral location of mglu_5 receptors, it might be expected that these would also be found on central terminations of C-fibres, but this does not appear to be the case^f. The role of spinal mglu_5 receptors is less clear, and a complicating factor might be the localization of this receptor on a variety of pre- and postsynaptic elements of either GABA- or non-GABA-containing neurones in the superficial dorsal horn^{b,c} (Fig. 1c). Spinal application of mglu_5 receptor antagonists might therefore produce variable effects that might depend partly on the relative distribution of drugs and physiological factors such as which neural circuits are in play in a given behavioural state. Thus, there is data that suggest little involvement of spinal cord mglu_5 receptors in either acute or chronic nociception^h, but

also evidence that spinal mglu_5 receptors might have a role in thermal hyperalgesiaⁱ or acute nociception^m. In the rat, there is less clear functional neuronal segregation between thermal and mechanical nociception supraspinally in the thalamus, but mglu_5 receptors are present in the thalamus^g, and direct application into the thalamus of MPEP selectively reduces acute thermal nociceptive responses of ventrobasal thalamic neuronesⁿ (Fig. 1i). These receptors might be an important target of systemic drugs because intravenous MPEP reduces such responses, but it is likely that mglu_5 receptors in other brain areas (e.g. the cerebral cortex and spinal cord) also contribute to these effects in the intact animal^o. Taken together, it is apparent that mglu_5 receptors provide a novel target for intervention in pain processes, and it is likely that mglu_5 receptor antagonism might be particularly valuable in specific pain types (e.g. inflammatory pain)^{a,h}. However, it should be remembered that other glutamate receptors, particularly mglu_1 receptors, have also been implicated in peripheral and central pain processes^{n,p,q}.

prominent anxiolytic-like activity of MPEP was seen in both the Vogel test and the four-plate test^{18,19}. Acquisition as well as expression of fear was shown to be inhibited by MPEP in the fear-potentiated startle test²⁰. Interestingly, acquisition but not expression of conditioned fear was also prevented by direct injection of MPEP into the lateral amygdala, directly linking mglu_5 receptors in the amygdala to fear conditioning²¹. In the Geller-Seifter test

the effects of MPEP were inconclusive because treatment with MPEP, up to a dose of 100 mg kg⁻¹, tended to increase the number of punished responses as well as the number of received shocks, but neither effect reached statistical significance²².

In the so-called unconditioned response tests MPEP exhibited anxiolytic-like activity in models of social anxiety (social exploration test), novelty-induced anxiety (marble burying test), anxiety in an

approach-avoidance conflict (elevated plus maze) and finally in a model of anticipatory anxiety (stress-induced hyperthermia)²². Together, these and other findings, including testing of closely related derivatives of MPEP (W.P.J.M. Spooren, unpublished), indicate that mglu_5 receptor antagonists have a very broad and potent anxiolytic-like activity in rodent models of anxiety (Table 2), with low potential to induce sedation or psychotomimetic effects²², although the

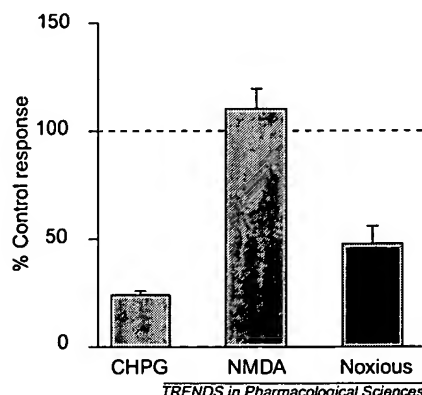


Fig. II. Reduction by iontophoretically applied 2-methyl-6-(phenylethynyl)-pyridine (MPEP) of responses of single thalamic neurones to iontophoretic application of the agonists (R,S)-2-chloro-5-hydroxyphenylglycine (CHPG) and NMDA, and to noxious thermal stimulation (Ref. n). Values are mean ± SEM (n = 6) percentages of the control responses recorded before MPEP application. Note that MPEP reduced CHPG and noxious responses but not NMDA responses.

References

- a Walker, K. *et al.* (2001) mGlu5 receptors and nociceptive function II. mGlu5 receptors functionally expressed on peripheral sensory neurones mediate inflammatory hyperalgesia. *Neuropharmacology* 40, 10–19
- b Valerio, A. *et al.* (1997) mGluR5 metabotropic glutamate receptor distribution in rat and human spinal cord: a developmental study. *Neurosci. Res.* 28, 49–57
- c Jia, H. *et al.* (1999) Metabotropic glutamate receptors in superficial laminae of the rat dorsal horn. *J. Comp. Neurol.* 410, 627–642
- d Berthele, A. *et al.* (1999) Distribution and developmental changes in metabotropic glutamate receptor messenger RNA expression in the rat lumbar spinal cord. *Dev. Brain Res.* 112, 39–53
- e Vidnyanszky, Z. *et al.* (1994) Cellular and subcellular localization of the mGluR5a metabotropic glutamate receptor in rat spinal cord. *NeuroReport* 6, 209–213
- f Romano, C. *et al.* (1996) Enhanced early developmental expression of the metabotropic glutamate receptor mGluR5 in rat brain: protein, mRNA splice variants, and regional distribution. *J. Comp. Neurol.* 367, 403–412
- g Neto, F.L. *et al.* (2000) Differential distribution of metabotropic glutamate receptor subtype mRNAs in the thalamus of the rat. *Brain Res.* 854, 93–105
- h Walker, K. *et al.* (2001) Metabotropic glutamate receptor subtype 5 (mGlu5) and nociceptive function. I. Selective blockade of mGlu5 receptors in models of acute, persistent and chronic pain. *Neuropharmacology* 40, 1–9
- i Dogrul, A. *et al.* (2000) Peripheral and spinal antihyperalgesic activity of SIB-1757, a metabotropic glutamate receptor (mGluR(5)) antagonist, in experimental neuropathic pain in rats. *Neurosci. Lett.* 292, 115–118
- j De Groot, J. *et al.* (2000) Peripheral glutamate release in the hindpaw following low and high intensity sciatic stimulation. *NeuroReport* 11, 497–502
- k Lawand, N.B. *et al.* (2000) Amino acid release into the knee joint: key role in nociception and inflammation. *Pain* 86, 69–74
- l Parpura, V. *et al.* (1995) Neuroligand-evoked calcium-dependent release of excitatory amino acids from Schwann cells. *J. Neurosci.* 15, 5831–5839
- m Bordi, F. and Ugolini, A. (2000) Involvement of mGluR(5) on acute nociceptive transmission. *Brain Res.* 871, 223–233
- n Salt, T.E. and Binns, K.E. (2000) Contributions of mGlu1 and mGlu5 receptors to interactions with *N*-methyl-D-aspartate receptor-mediated responses and nociceptive sensory responses of rat thalamic neurons. *Neuroscience* 100, 375–380
- o Binns, K.E. and Salt, T.E. (2001) Actions of the systemically active metabotropic glutamate antagonist MPEP on sensory responses of thalamic neurones. *Neuropharmacology* 40, 639–644
- p Hargett, G.L. *et al.* (2000) Presynaptic control of primary afferents by mGluR1α receptors. *Soc. Neurosci.* 26, 949
- q Young, M.R. *et al.* (1998) Antisense ablation of type I metabotropic glutamate receptor mGluR1 inhibits spinal nociceptive transmission. *J. Neurosci.* 18, 10180–10188

scope of potential CNS effects needs to be explored much further. Finally, although MPEP was inactive in the swim test (Table 2), MPEP shortened the immobility time in the tail suspension test¹⁸. These data suggest that MPEP might also have antidepressant-like activity, which remains to be further investigated.

Epilepsy

The role of distinct mglu receptor subtypes in epilepsy is at present not clear.

However, intracerebroventricular application of CHPG in mice was shown to induce clonic seizures that were suppressed by MPEP or SIB1893 (Ref. 23). Both compounds also suppressed sound-induced seizures in DBA-2 mice and clonic seizures in the *lethargic* mouse²³. Together, these data indicate that mglu₅ receptor antagonists exhibit anticonvulsant activity, which clearly needs further characterization in additional studies.

Neuroprotection

It has been shown that noncompetitive mglu₅ receptor antagonists exhibit neuroprotection against excitotoxic-induced degeneration *in vitro* and *in vivo*, as well as against β-amyloid-induced toxicity *in vitro*^{8,24,25}. However, the role of mglu₅ receptors in neuroprotection is controversial in view of the varying concentrations of MPEP applied in different models. Given the relatively good selectivity of MPEP (Ref. 7) effects observed at concentrations close to the IC₅₀ at mglu₅ receptors (e.g. partial inhibition of β-amyloid-induced toxicity and excitotoxicity induced by a short pulse of NMDA)²⁴ are indeed likely to reflect specific action at mglu₅ receptors whereas neuroprotection achieved at very high concentrations of MPEP (20 μM and 200 μM, 1000–10 000-fold above its IC₅₀, respectively) might instead reflect a combined action of MPEP at mglu₅ and additional receptors, such as NMDA receptors^{8,25}.

Parkinson's disease

The expression of mglu₅ receptors in the striatum and subthalamic nucleus suggests that antagonists might reduce the overactive glutamate-mediated pathways in the basal ganglia in Parkinson's disease. mglu₅ receptors have been implicated as major players in the excitatory drive to the subthalamic nucleus from glutamatergic afferents¹⁰. Indeed, in the unilateral 6-hydroxy-dopamine (6-OHDA) rat rotation model of Parkinson's disease, (acute) MPEP increased the number of net rotations (Table 2). However, the effect was marginal and consisted of an ipsilateral bias in spontaneous turning rather than a clear turning response²⁶. In additional studies it was shown that MPEP inhibited dopamine-mediated rotations. The underlying mechanism of these findings is at present unclear. However, the interaction of MPEP with dopamine clearly awaits further elucidation in additional studies using distinct models of Parkinson's disease as well as applying a much wider dose range of MPEP.

Interestingly, in a conditioned reaction-time task in the rat, chronic but not acute treatment with MPEP significantly and gradually reduced the motor deficits induced by bilateral lesions of the nigrostriatal pathway

Table 2. Effects of the mglu₅ receptor antagonist MPEP in animal models of distinct nervous system disorders^{a,b}

Indication and model	Species	Tested dose range (mg kg ⁻¹) ^c	Effective dose range (mg kg ⁻¹)	Route of administration	Refs
Pain					
Tail flick	Rat	10–100	–	p.o.	27
FCA-induced mechanical hyperalgesia	Rat	10–100	30–100	p.o.	27,28
Carageenin-induced mechanical hyperalgesia	Rat	0.03–0.3 µmole	0.03–0.3 µmole	i.pl.	27
Neuropathic hyperalgesia	Rat	3–100	30–100	p.o.	27,28
Anxiety					
EPM	Rat	0.1–30	0.1–10 and 10–30	p.o.	18,19,22
Social exploration	Rat	0.003–10	0.3–1	p.o.	22
SIH	Mouse	1.5–30	15–30	p.o.	22
Marble burying	Mouse	1.5–30	7.5–30	p.o.	22
Vogel conflict	Rat	0.3–10	1–10	i.p.	18,19
Geller-Seifter	Rat	10–100	–	p.o.	22
Four-plate test	Mouse	3–30	30	i.p.	18,19
Fear potentiated startle: acquisition	Rat	0.3–30	3–30	p.o.	20
expression		0.3–30	30	p.o.	
Depression					
Swim test	Rat	0.1–30	–	i.p.	18
Tail suspension	Mouse	0.1–20	1–20	i.p.	18
Parkinson's disease					
6-OHDA model	Rat	7.5–30	30	p.o.	26
Side-effects					
PPI	Rat	1–10	–	p.o.	22
Rotarod	Rat	7.5–300	–	p.o.	26
Spontaneous locomotor activity:	Mouse				22
horizontal activity		0.01–100	–	p.o.	
vertical activity		0.01–100	100 (↓)	p.o.	

^aAbbreviations: EPM, elevated plus-maze; FCA, Freund's complete adjuvant; i.p., intra-peritoneal; i.pl., intra-plantar; mglu, metabotropic glutamate; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; 6-OHDA, 6-hydroxydopamine; p.o., oral; PPI, prepulse inhibition; SIH, stress-induced hyperthermia.

^bSymbols: –, no effect; ↓ decrease.

^cDose is given as mg kg⁻¹ except for FCA-induced mechanical hyperalgesia.

(M. Amalric *et al.*, unpublished).

Comparable results were obtained with chronic L-3,4-dihydroxyphenylalanine (L-DOPA) treatment. Subsequent studies in the unilateral 6-OHDA rat rotation model confirmed sensitization to MPEP following a sub-chronic (7 days) application resulting in a (limited) rotational response rather than an ipsilateral bias in spontaneous turning (W.P.J.M. Spooren *et al.*, unpublished). Furthermore, MPEP was found to counteract haloperidol-induced muscle rigidity and akinesia (A. Pilc *et al.*, unpublished). Accordingly, it appears that mglu₅ receptor antagonists have minor motor stimulating properties in models of Parkinson's disease but might improve rigidity and bradykinesia and thus provide an interesting new approach for the symptomatic treatment of Parkinson's disease.

Concluding remarks

The identification of the first subtype-selective mglu₅ receptor antagonists has fostered important progress in the understanding of the role of mglu₅ receptors in neurological and behavioural disorders. On the basis of published studies, a clear indication for the potential therapeutic use of mglu₅ receptor antagonists seems to be in chronic pain that arises from inflammatory conditions. Furthermore, additional potential indications are anxiety, epilepsy and chronic neurodegenerative diseases such as Parkinson's disease. Other potential applications such as asthma and bowel disorders, which involve peripherally expressed mglu₅ receptors, are only beginning to emerge and await further exploration. The next few years will witness major advances in the development of noncompetitive antagonists for mglu₅

receptors as well as other mglu receptors, and will bring further understanding in the role of these receptors in (patho)physiological processes.

Acknowledgements

We would like to thank M. Amalric (CNRS, Marseille) for critical reading of the manuscript, R. Shigemoto (National Institute for Physiological Sciences, Okazaki) and J. Winter (Novartis Institute for Medical Sciences, London) for kindly permitting the reproduction of immunohistochemical stainings of mglu₁ and mglu₅ receptors, and M. Amalric and A. Pilc (Institute of Pharmacology, Krakow) for communication of unpublished data.

References

- Conn, J. and Pin, J.P. (1997) Pharmacology and functions of metabotropic glutamate receptors. *Annu. Rev. Pharmacol. Toxicol.* 37, 205–237
- Martin, L.J. *et al.* (1992) Cellular localization of a metabotropic glutamate receptor in rat brain. *Neuron* 9, 259–270
- Shigemoto, R. and Mizuno, N. (2000) Metabotropic glutamate receptors – immunocytochemical and *in situ* hybridization analysis. In *Handbook of Chemical Neuroanatomy: Metabotropic Glutamate Receptors: Immunocytochemical and in situ Hybridization Analyses* (Vol. 18) (Ottersen, O.P. and Storm-Mathisen, J., eds), pp. 63–98, Elsevier
- Schoepp, D.D. *et al.* (1999) Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology* 38, 1431–1476
- Doherty, A.J. *et al.* (2000) A novel, competitive mGlu(5) receptor antagonist (LY344545) blocks DHPG-induced potentiation of NMDA responses but not the induction of LTP in rat hippocampal slices. *Br. J. Pharmacol.* 131, 239–244
- Varney, M. *et al.* (1999) SIB-1757 and SIB-1893: selective, noncompetitive antagonists of metabotropic glutamate receptor type 5 (mGluR5). *J. Pharmacol. Exp. Ther.* 290, 170–181
- Gasparini, F. *et al.* (1999) 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. *Neuropharmacology* 38, 1493–1504
- O'Leary, D.M. *et al.* (2000) Selective mGluR5 antagonists MPEP and SIB-1893 decrease NMDA or glutamate-mediated neuronal toxicity through actions that reflect NMDA receptor antagonism. *Br. J. Pharmacol.* 131, 1429–1437
- Turner, J.P. and Salt, T.E. (2000) Synaptic activation of the group I metabotropic glutamate receptor mGlu1 on the thalamocortical neurons of the rat dorsal lateral geniculate nucleus *in vitro*. *Neuroscience* 100, 493–505
- Awad, H. *et al.* (2000) Activation of metabotropic glutamate receptor 5 has direct excitatory effects and potentiates NMDA receptor currents in neurons of the subthalamic nucleus. *J. Neurosci.* 20, 7871–7879
- Salt, T.E. and Binns, K.E. (2000) Contributions of mGlu1 and mGlu5 receptors to interactions with N-methyl-D-aspartate receptor-mediated responses and nociceptive sensory responses of rat thalamic neurons. *Neuroscience* 100, 375–380

- 12 Doherty, A.J. *et al.* (1997) (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) activates mGlu5, but not mGlu1, receptors expressed in CHO cells and potentiates NMDA responses in the hippocampus. *Neuropharmacology* 36, 265–267
- 13 Alagarsamy, S. *et al.* (1999) Activation of NMDA receptors reverses desensitization of mGluR5 in native and recombinant systems. *Nat. Neurosci.* 2, 234–240
- 14 Bond, A. and Lodge, D. (1995) Pharmacology of metabotropic glutamate receptor-mediated enhancement of responses to excitatory and inhibitory amino acids on rat spinal neurones *in vivo*. *Neuropharmacology* 34, 1015–1023
- 15 Ugolini, A. *et al.* (1999) Potentiation of NMDA and AMPA responses by the specific mGluR5 agonist CHPG in spinal cord motoneurons. *Neuropharmacology* 38, 1569–1576
- 16 Jia, Z. *et al.* (1998) Selective abolition of the NMDA component of long-term potentiation in mice lacking mGluR5. *Learn. Mem.* 5, 331–343
- 17 Salt, T.E. and Eaton, S.A. (1994) The function of metabotropic excitatory amino acid receptors in synaptic transmission in the thalamus: studies with novel phenylglycine antagonists. *Neurochem. Int.* 24, 451–458
- 18 Tatarczynska, E. *et al.* (2001) Potential anxiolytic and antidepressant-like effects of MPEP, a potent, selective and systemically active mGlu5 receptor antagonist. *Br. J. Pharmacol.* 132, 1423–1430
- 19 Klodzinska, A. *et al.* (2000) Anxiolytic-like effects of group I metabotropic antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) in rats. *Pol. J. Pharmacol.* 52, 463–466
- 20 Schultz, B. *et al.* The metabotropic glutamate receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) blocks fear conditioning in rats. *Neuropharmacology* (in press)
- 21 Fendt, M. *et al.* (2000) Injections of the metabotropic glutamate receptor antagonist MPEP into the lateral amygdala prevent acquisition of conditioned fear in rats. *Soc. Neurosci.* 26, 200
- 22 Spooren, W.P.J.M. *et al.* (2000) Anxiolytic-like effects of the prototypical metabotropic glutamate receptor 5 (mGlu₅) antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) in rodents. *J. Pharmacol. Exp. Ther.* 295, 1267–1275
- 23 Chapman, A.G. *et al.* (2000) Anticonvulsant activity of two metabotropic glutamate group I antagonists selective for the mGlu5 receptor: 2-methyl-6-(phenylethynyl)-pyridine (MPEP), and (E)-6-methyl-2-styryl-pyridine (SIB 1893). *Neuropharmacology* 39, 1567–1574
- 24 Bruno, V. *et al.* (2000) Selective blockade of metabotropic glutamate receptor subtype 5 is neuroprotective. *Neuropharmacology* 39, 2223–2230
- 25 Movsesyan, V. *et al.* (2001) mGluR5 antagonists 2-methyl-6-(phenylethynyl)-pyridine and (E)-2-methyl-6-(2-phenylethenyl)-pyridine reduce traumatic neuronal injury *in vitro* and *in vivo* by antagonizing N-methyl-D-aspartate receptors. *J. Pharmacol. Exp. Ther.* 296, 41–47
- 26 Spooren, W.P.J.M. *et al.* (2000) Effects of the prototypical mGlu(5) receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine on rotarod, locomotor activity and rotational responses in unilateral 6-OHDA-lesioned rats. *Eur. J. Pharmacol.* 406, 403–410
- 27 Walker, K. *et al.* (2001) Metabotropic glutamate receptor subtype 5 (mGlu5) and nociceptive function. I. Selective blockade of mGlu5 receptors in models of acute, persistent and chronic pain. *Neuropharmacology* 40, 1–9
- 28 Walker, K. *et al.* (2001) mGlu5 receptors and nociceptive function II. mGlu5 receptors functionally expressed on peripheral sensory neurones mediate inflammatory hyperalgesia. *Neuropharmacology* 40, 10–19

Chemical name

LY344545: (1R,2S)-2-[(R)-1-amino-1-carboxy-2-(9H-thioxanthen-9-yl)-ethyl]-cyclopropanecarboxylic acid

LY393675: 3-(S)-1-amino-1-carboxy-2-(9H-thioxanthen-9-yl)-ethyl]-cyclobutanecarboxylic acid

SIB1757: 6-methyl-2-(phenylazo)-3-pyridinol

SIB1893: (E)-2-methyl-6-(2-phenylethenyl)pyridine

Will P.J.M. Spooren*

Fabrizio Gasparini

Rainer Kuhn

Novartis Pharma AG, Nervous System Research, CH-4002 Basel, Switzerland.

*e-mail:

willibrordus.spooren@pharma.novartis.com

Thomas E. Salt

Institute of Ophthalmology, University College London, London, UK EC1V 9EL.

Meeting Report

Histamine receptors are finally 'coming out'

Rob Leurs, Takehiko Watanabe and Henk Timmerman

The International Sendai Histamine Symposium *Histamine Research in the New Millennium* was held in Sendai, Japan on 22–25 November 2000.

The *Histamine Research in the New Millennium Symposium*, which covered various aspects of the histamine-mediated system, was one of the most exciting meetings in this field in past years, and was enjoyed by 300 attendants from 25 countries. Important information on histamine H₁ and H₂ receptors was reported, but the main reason for this enthusiasm was the wealth of new information on several new histamine receptors (Table 1).

Cloning and characterization of the H₃ receptor

Following the cloning of the human histamine H₃ receptor by Lovenberg *et al.*

in 1999 (Ref. 1), the field of histamine receptors has gained considerable interest. At the meeting Tim Lovenberg (R.W. Johnson Pharmaceutical Research Institute, San Diego, CA, USA) briefly reviewed the cloning of the human and rat H₃ receptor cDNAs as part of their ongoing effort to identify orphan G-protein-coupled receptors. The two receptors exhibit a 97% homology in the transmembrane (TM) domains^{1,2}, but surprisingly they display a significant difference in affinity for some H₃ receptor ligands (e.g. thioperamide shows a tenfold preference for the rat receptor). Cloning of the mouse H₃ receptor allowed Lovenberg and co-workers to generate H₃ receptor knockout mice. These animals are viable and fertile, but less 'active' at all times. The mice are currently undergoing further testing and will provide interesting new model systems to learn more about the

(patho)physiological role of the H₃ receptor.

Investigations by the groups of Rob Leurs (Leiden/Amsterdam Center for Drug Research, Amsterdam, The Netherlands) and Jean-Charles Schwartz (INSERM, Paris, France) revealed that Thr119 and Ala122 are fully responsible for the observed relative low affinity of thioperamide, iodophenpropit and ciproxyfan at the human H₃ receptor. The same two groups also reported on the high level of constitutive activity of the human and rat H₃ receptor, resulting in a reclassification of a variety of H₃ receptor antagonists. Burimamide, originally found to be an H₃ receptor antagonist³, surprisingly acts as an agonist at the recombinant H₃ receptor, whereas compounds such as thioperamide, clobenpropit and ciproxyfan behave as inverse agonists. The constitutive activity



PERGAMON

Progress in Neurobiology Vol. 59, pp. 55 to 79, 1999
 © 1999 Elsevier Science Ltd. All rights reserved
 Printed in Great Britain
 0301-0082/99/\$ - see front matter

PII: S0301-0082(98)00095-1

GROUP I METABOTROPIC GLUTAMATE RECEPTORS: IMPLICATIONS FOR BRAIN DISEASES

FABIO BORDI* and ANNAROSA UGOLINI

Pharmacology Department, GlaxoWellcome Medicine Research Centre, 37100 Verona, Italy

(Received 26 October 1998)

Abstract—Glutamate is the major excitatory neurotransmitter in the brain and plays a unique role in a variety of central nervous system (CNS) functions. The discovery of the metabotropic receptors (mGluRs), a family of G-protein coupled receptors that can be activated by glutamate, has led to an impressive number of studies in recent years aimed at understanding their biochemical, physiological and pharmacological characteristics.

The eight mGluRs now known are divided into three groups according to their sequence homology, signal transduction mechanisms, and agonist selectivity. Group I mGluRs include mGluR₁ and mGluR₃, which are linked to the activation of phospholipase C; Groups II and III include all others and are negatively coupled to adenylyl cyclases.

The availability in recent years of agents selective for Group I mGluRs has made possible the study of the physiological roles of these receptors in the CNS. In addition to mediating glutamatergic neurotransmission, Group I mGluRs can modulate other neurotransmitter receptors, including GABA and the ionotropic glutamate receptors.

Group I mGluRs are involved in many CNS functions and may participate in a variety of disorders such as pain, epilepsy, ischemia, and chronic neurodegenerative diseases. This class of receptor may provide important pharmacological therapeutic targets and elucidating its functions will be relevant to develop new treatments for neurological and psychiatric disorders in which glutamatergic neurotransmission is abnormally regulated.

In this review anatomical, physiological and pharmacological results are presented with a special emphasis on the role of Group I mGluRs in functional and pathological processes. © 1999 Elsevier Science Ltd. All rights reserved

CONTENTS

1. Introduction	56
2. Pharmacology of the Group I mGluRs	56
3. Distribution of Group I mGluRs in the mammalian brain	58
4. Neurophysiological role of Group I mGluRs	58
4.1. Neuronal excitability	58
4.2. Modulation of synaptic transmission	60
4.2.1. Presynaptic inhibition	60
4.2.2. Modulation of GABA	60
4.2.3. Modulation of other neurotransmitters	60
4.2.4. Modulation of ionotropic GluRs	61
4.3. Long-term regulation of synaptic efficacy	62
4.3.1. LTP and memory	62
4.3.2. LTD	66
5. Group I mGluRs in pathological conditions	66
5.1. Role of Group I mGluRs in nociception	66
5.2. Role of Group I mGluRs in epilepsy	68
5.3. Role of Group I mGluRs in neurodegeneration	69
5.3.1. Brain ischemia	69
5.3.2. Huntington's and Parkinson's diseases	70
5.3.3. Alzheimer's disease	71
5.4. Role of Group I mGluRs in psychiatry disorders	71
6. Conclusions	71
Acknowledgements	72
References	72

* Corresponding author. Tel.: +39/045/921-8845; Fax: +39/045/921-8153; e-mail: fb23261@GlaxoWellcome.co.uk

ABBREVIATIONS

4CPG	(S)-4-Carboxyphenylglycine	EPSP	Excitatory postsynaptic potential
4C3HPG	(S)-4-Carboxy-3-hydroxyphenylglycine	iGluR	Ionotropic glutamate receptor
AC	Adenylyl cyclase	IP ₃	Inositol triphosphate
ACPD	1S,3R-1-Amino-1,3-cyclopentanedicarboxylate	LTD	Long-term depression
AIDA	(RS)-1-Aminoindan-1,5-dicarboxylic acid	LTP	Long-term potentiation
AMPA	(S)- α -Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid	mGluR	Metabotropic glutamate receptor
CHPG	(RS)-2-Chloro-5-hydroxyphenylglycine	MCPG	α -Methyl-4-carboxyphenylglycine
DAG	Diacylglycerol	NMDA	N-Methyl-D-aspartate
DHPG	3,5-Dihydroxyphenylglycine	PKC	Protein kinase C
EPSC	Excitatory postsynaptic current	PLC	Phospholipase C

1. INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) and plays an important role in a number of CNS functions (Monaghan *et al.*, 1989; Hollmann and Heinemann, 1994). The receptors that mediate its action have been divided into two broad categories, termed ionotropic receptors, which contain glutamate-gated cation channels, and metabotropic receptors, which are coupled to GTP-binding proteins (G proteins) and linked to the activation of phospholipase C (PLC) or the inhibition or activation of adenylyl cyclases (AC).

The metabotropic receptors (mGluRs) were discovered only in the 1980s when it was shown that glutamate and other excitatory amino acids like quisqualate and ibotenate could stimulate phosphoinositide (PI) turnover (Sladeczek *et al.*, 1985; Nicoletti *et al.*, 1986; Sugiyama *et al.*, 1987) or lead to mobilization of intracellular Ca^{2+} in different cell types of the CNS (Mayer and Miller, 1990; Irving *et al.*, 1990). Soon after, the first cDNA clone of a mGluR subtype was isolated by functional expression cloning (Masu *et al.*, 1991; Houamed *et al.*, 1991). This subtype is now known as mGluR₁. In the following years the cloning effort rapidly led to the discovery of seven more different subtypes, termed mGluR₂ to mGluR₈ [Tanabe *et al.* (1992); Abe *et al.* (1992); Nakanishi and Masu (1994) for a review]. Based on sequence homology, signal transduction mechanisms and agonist selectivity, the eight mGluRs can be divided into three groups (Nakanishi, 1994; Pin and Duvoisin, 1995). Group I includes mGluR₁ (with its splice variants a, b, c and d) and mGluR₅ (a and b), which are positively coupled to PLC and lead to an increase in diacylglycerol (DAG) and inositol triphosphate (IP₃), and in some cases to an activation of AC (Aramori and Nakanishi, 1992); Groups II and III include all others and lead to a decrease in forskolin-stimulated AC [Conn and Pin (1997) for review].

Over the recent years the Group I mGluRs have been extensively studied in experimental animals, leading to an appreciation of their great importance in the CNS. Both mGluR₁ and mGluR₅ are present in a number of key CNS structures including the hippocampus, cortex, thalamus and cerebellum and the involvement of these receptors in a variety of disorders including epilepsy, ischemia, pain and neurodegenerative diseases is beginning to emerge.

Drugs that act through the mGluRs may have the capability to modulate glutamatergic synapses in a selective manner without the potential side-effects that had limited the clinical usefulness of the ionotropic glutamate agents (Olney, 1994). This new class of receptors could provide unique opportunities for designing more selective therapies.

Trans-amino-cyclopentane-dicarboxylate (*trans*-ACPD) was the first compound shown to activate mGluRs selectively (Palmer *et al.*, 1989) but lack of mGluR subtype-specific drugs made it difficult to draw conclusions about the physiological roles of the various metabotropic receptor groups. Thanks to the synthesis of new selective agonists and antagonists in recent years it is becoming possible to distinguish among the mGluR groups and much improvement has been made in the understanding of the biochemical, physiological and pharmacological properties of the Group I mGluRs (Watkins and Collingridge, 1994; Roberts, 1995; Conn and Pin, 1997). In this review we will examine this new and still somewhat controversial topic, emphasising the role of Group I mGluRs in functional and pathological processes.

2. PHARMACOLOGY OF THE GROUP I mGLURs

Group I mGluRs stimulate PLC and PI hydrolysis [see Conn and Pin (1997) for a review], and in some cases these receptors can couple to AC (Aramori and Nakanishi, 1992).

Cloned mGluRs expressed in heterologous mammalian systems have been used to characterize the activity of selective compounds on mGluR subtypes. Using predominately mGluR_{1a} or mGluR_{5a} splice variants, the pharmacological profile of Group I mGluRs has been determined (Conn and Pin, 1997). In every cell line tested, quisqualate is the most potent agonist of these receptors followed by ibotenate and glutamate, the presumed endogenous transmitter (Aramori and Nakanishi, 1992; Nakanishi *et al.*, 1994; Joly *et al.*, 1995). The rank of potency of the agonists for Group I mGluRs was found to be: quisqualate > 3,5-dihydroxyphenylglycine (DHPG) = glutamate > 1S,3R-1-amino-1,3-cyclopentanedicarboxylate (ACPD) = ibotenate > (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I) > 3-hydroxyphenylglycine (3-HPG) > *trans*-azetidine-2,4-dicarboxylate (*t*-ADA) (Pin and Duvoisin,

1995; Conn and Pin, 1997). The Group I mGluR specific agonists are DHPG, 3-HPG and *t*-ADA, which are devoid of activity in other mGluR groups (Ito *et al.*, 1992; Schoepp *et al.*, 1994; Brabet *et al.*, 1995; Hayashi *et al.*, 1994). A new phenylglycine derivative, (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG), has been recently reported to activate only mGluR₅, but not mGluR₁ in transfected cells. This is the first compound available to distinguish pharmacologically the two members of the Group I class, although it is not very potent (Doherty *et al.*, 1997).

The discovery in 1993 of phenylglycine compounds as antagonists of mGluRs made possible to test the selectivity of the various mGluRs in both heterologous and neuronal systems. The first antagonist described for Group I mGluRs was α -methyl-4-carboxyphenylglycine (MCPG) (Birise *et al.*, 1993; Eaton *et al.*, 1993a; Watkins and Collingridge, 1994). MCPG, however, is also an antagonist of Group II mGluRs (Hayashi *et al.*, 1994). Substances related to phenylglycine have been synthesized since then, with (*S*)-4-carboxyphenylglycine (4CPG) and (*S*)-4-carboxy-3-hydroxyphenylglycine (4C3HPG) found to be the most potent Group I mGluRs antagonists, although they also have agonist effects on Group II receptors (Hayashi *et al.*, 1994; Thomsen *et al.*, 1994). Some of these compounds were found to be selective for mGluR₁ over mGluR₅, like LY367385 (Clark *et al.*, 1997). Even MCPG and 4CPG show a much better sensitivity for mGluR₁ than mGluR₅ in transfected cells (Joly *et al.*, 1995). Very recently, other compounds with an improved potency for the Group I mGluRs have been synthesized. These include (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) (Pellicciari *et al.*, 1995; Moroni *et al.*, 1997), (*S*)-(+)-2,3'-carboxybicyclo[1.1.1]pentylglycine (CBPG) (Pellicciari *et al.*, 1996), and ethyl 7-(hydroxyimino)cyclopropan[b]chromen-1 α -carboxylate (EtCCC) (Annoura *et al.*, 1996).

Most of the results obtained in clonal cell lines have been confirmed in native neuronal systems, such as brain slices or cultured cells, where the stimulation of PI hydrolysis is measured, or in spinal cord, where the depolarization induced in motoneurons by specific agonists can be inhibited by selective antagonists. Table 1 shows the potency of the agonists and antagonists that act at the Group I mGluRs measured in transfected cell lines or in native systems.

Since mGluR₁ is highly expressed while mGluR₅ is almost absent in the cerebellum [Masu *et al.* (1991); Shigemoto *et al.* (1993) see below], this structure is particularly suitable for distinguishing between the two Group I mGluRs (Toms *et al.*, 1995; Chavis *et al.*, 1995; Batchelor *et al.*, 1997). Cultured granule cells from rat cerebellum appear to be an excellent system for investigating selective compounds in a native environment. Using this system to assay mGluR₁ responses coupled to PI hydrolysis, Toms *et al.* (1995) obtained similar pharmacological results to those reported for cloned mGluR₁. The pharmacological characteristics of mGluR₁ can also be studied in the intact cerebellar slice, where mGluR-mediated excitatory postsynaptic potentials (EPSP) are recorded from Purkinje cells (Batchelor *et al.*, 1994, 1997).

Another useful preparation for characterizing Group I mGluRs is the neonatal rat spinal cord. Postsynaptic depolarization of motoneurons induced by specific agonists can be recorded electrophysiologically from the ventral roots (Watkins and Collingridge, 1994). This depolarization is insensitive to ionotropic glutamate receptor (iGluR) antagonists, but it is inhibited in a dose-dependent fashion by specific Group I mGluR antagonists (Birise *et al.*, 1993; Jane *et al.*, 1993; Ugolini *et al.*, 1997).

Table 1.

	Cloned cells		Neuronal systems		
	mGluR ₁	mGluR ₅	Hippocampal or cortical cells	Cerebellar granule cells	Neonatal spinal cord
<i>Agonist (EC₅₀ values in mM)</i>					
Glutamate	9–13	3–10		50 ^{††}	
Quisqualate	0.2–3	0.03–0.3		2 ^{††}	
(1 <i>S</i> ,3 <i>R</i>)-ACPD	10–80	5–7	47.2 [¶]	102 ^{††}	58.1 ^{§§}
DHPG			27.6 [¶]		
3-HPG	68–100	14–35	98 ^{**}		
<i>t</i> -ADA	190	30			
CHPG	Not active*	750*			
<i>Antagonist (IC₅₀ or K_D values in mM)</i>					
MCPG	40–200	> 200	130 ^{††}	243 ^{††}	341 ^{††} ; 243 ^{§§}
4CPG	15–65	> 500	35 ^{††}	51 ^{††}	208 ^{††}
4C3HPG	10–40	?	345 ^{**}	41 ^{††}	
AIDA	214 [†]	> 1000 [†]			
EtCCC	23 [‡]		40 ^{††}		
LY367385	8.8 [§]	> 100 [§]			

Values are taken from the following references: *Doherty *et al.* (1997); †Moroni *et al.* (1997); ‡Annoura *et al.* (1996); §Clark *et al.* (1997); ¶Schoepp *et al.* (1994); **Birise *et al.* (1993); ††Batchelor *et al.* (1997); ‡‡Toms *et al.* (1995); §§Boxall *et al.* (1996); †††Jones *et al.* (1993). All other values are taken from Conn and Pin (1997).

3. DISTRIBUTION OF GROUP I mGluRs IN THE MAMMALIAN BRAIN

With the isolation of the first cDNA for a mGluR, the mGluR₁, it became possible to study the expression of these receptors in the CNS (Masu *et al.*, 1991). Distribution of the mRNA for mGluR₁ by *in situ* hybridization revealed an abundant expression of this receptor in hippocampal neurons and cerebellar Purkinje cells (Shigemoto *et al.*, 1992; Fotuhi *et al.*, 1993). In the cerebellum, mGluR₁ is distributed in cell bodies and dendrites of Purkinje, stellate and some Golgi cells, as described by immunoreactivity for mGluR_{1a} (Martin *et al.*, 1992; Baude *et al.*, 1993; Hampson *et al.*, 1994). No mGluR₅, on the other hand, is found in the cerebellum (Shigemoto *et al.*, 1993), except for a small signal in the granule cell layer but not in the Purkinje cells (Romano *et al.*, 1995). The strong presence of mGluR₁ in the cerebellum correlates well with the severe motor coordination syndrome characterized by ataxia and tremor and with the neurophysiological abnormalities obtained with the mGluR₁-deficient mice (Conquet *et al.*, 1994; Aiba *et al.*, 1994b).

Other areas of expression of mGluR₁ are the olfactory bulb, the amygdala, the thalamus, and the basal ganglia (Martin *et al.*, 1992; Baude *et al.*, 1993; Hampson *et al.*, 1994). The presence of this receptor in these areas has inspired study of the potential role of Group I mGluRs in extrapyramidal motor diseases like Huntington Chorea or Parkinson's Disease (see later).

In the hippocampus, both CA1 and CA3 fields are heavily labeled by antibodies to mGluR_{1a}, but this immunoreactivity is present in 'non-principal' neurons and is concentrated in the postsynaptic membrane at the periphery of synaptic junctions (Baude *et al.*, 1993). Unlike antibodies to glutamate ionotropic receptors that label sites within the synaptic junctions (Nusser *et al.*, 1994, 1995), the antibodies to mGluR_{1a} label sites that are at the edge of the synapse (Baude *et al.*, 1993). Because of the special segregation of ionotropic receptors and mGluRs, it has been suggested that they may respond differently to glutamate stimulation; the ionotropic receptors respond to glutamate under normal presynaptic stimulation, whereas the perisynaptic mGluR₁ is involved in excitatory responses evoked only by strong presynaptic stimulation. This anatomical distribution suggests a role for the mGluRs in synaptic function that is different from that of the ionotropic receptors which, mediating fast signals at glutamergic synapses, are close to release sites. The postsynaptic metabotropic receptors situated at the periphery would appear to have a role of delayed activation (Baude *et al.*, 1993). Although these observations have been made using the mGluR_{1a} splice variant, the regional pattern of the immunoreactivity for this splice variant suggests that its distribution correlates with the mRNA coding for all mGluR₁ (Shigemoto *et al.*, 1992). Interestingly, recent studies have confirmed this perisynaptic location in the hippocampus also for mGluR₅ and it may be a general feature of all G-protein receptors (Luján *et al.*, 1996).

The hippocampus of the rat (Shigemoto *et al.*, 1993; Romano *et al.*, 1995) and the human (Blümcke *et al.*, 1996) contain many neurons that express mGluR₅. In the CA1 region of the rat, mGluR₅ is present in both pre- and postsynaptic membranes (Romano *et al.*, 1995). However, while most CA3 pyramidal and granule cells express both mGluR₅ and mGluR₁, CA1 pyramidal cells express only mGluR₅ (Luján *et al.*, 1996). This finding fits nicely with the neurophysiological results demonstrating that LTP can be induced in CA1 region in mice lacking mGluR₁ (Conquet *et al.*, 1994), but not in mice lacking mGluR₅ [Lu *et al.* (1997); see later].

Group I mGluRs are also abundant in the spinal cord (Vidnyánszky *et al.*, 1994; Valerio *et al.*, 1997a,b). mGluR_{5a} antibody staining is heavy in laminae I–II of the dorsal horn of the rat at the level of dendrites and perikarya and is found around the postsynaptic membrane in a peri- or extrasynaptic location similar to that seen in the hippocampus (Vidnyánszky *et al.*, 1994). This distribution of mGluR₅ is also seen in the human spinal cord (Valerio *et al.*, 1997a). Rat spinal cord expresses high levels of mRNA encoding mGluR_{1a} as well (Valerio *et al.*, 1997b). The presence of both mGluR₁ and mGluR₅ in the dorsal laminae of the spinal cord strongly suggests an important role for these receptors in the control of nociceptive transmission [Corsi *et al.* (1997) see later].

4. NEUROPHYSIOLOGICAL ROLE OF GROUP I mGluRs

Metabotropic glutamate receptor agonists have a wide variety of actions in the CNS that can be mediated by voltage- and ligand-gated ion channels. Substances that activate Group I mGluRs cause neuronal depolarization and excitation while antagonists of these receptors inhibit depolarization and excitation. In addition, activation of these receptors can modulate synaptic transmission by regulating GABA release or by interfering with iGluRs. Recent evidence has also shown the importance of Group I mGluRs in modulating other brain neurotransmitter systems.

4.1. Neuronal Excitability

Application of the broad spectrum mGluR agonist ACPD produces an array of physiological effects, both inhibitory and excitatory (Schoepp *et al.*, 1990; Conn and Pin, 1997). Group I mGluRs, however, seem to involve primarily postsynaptic excitatory effects. In the CA1 area of the hippocampus, activation of mGluRs with ACPD leads to depolarization, increase of input resistance, and reduction in spike frequency adaptation (Charpak *et al.*, 1990; Desai and Conn, 1991; Desai *et al.*, 1992). These effects have been attributed to activation of Group I mGluRs because they are also produced by the specific agonist DHPG (Gereau and Conn, 1995a) and are blocked by the antagonists MCPG and 4CPG (Davies *et al.*, 1995). In neocortical neurons, the reduction in spike frequency adaptation caused by ACPD or quisqualate and blocked by

MCPG is not prevented by protein kinase C (PKC) or protein kinase A (Burke and Hablitz, 1996).

The increase in neuronal excitability caused by activation of Group I mGluRs is produced primarily by modulation of potassium channels [for review, Gerber and Gähwiler (1994); Glaum and Miller (1994)]. ACPD induces net inward currents by inhibiting K^+ conductances in neurons in hippocampus (Charpak *et al.*, 1990; Crépel *et al.*, 1994; Gereau and Conn, 1995a), amygdala (Womble and Moises, 1994), nucleus of the tractus solitarius (Glaum and Miller, 1992), and hypothalamus (Schrader and Tasker, 1997). This effect is blocked by Group I mGluR selective antagonists (Guérineau *et al.*, 1995; Gereau and Conn, 1995a; Schrader and Tasker, 1997). In the CA3 region of the hippocampus the reduction of K^+ conductance in response to mGluR activation is not mediated by PKC or protein kinase A and it exhibits a pronounced voltage sensitivity which could be important in physiological processes such as long-lasting changes in cellular excitability or persistent modification of synaptic efficacy (Gerber and Gähwiler, 1994; Lüthi *et al.*, 1997).

Interestingly, a decrease in K^+ conductance has been hypothesized to account for the control of corticothalamic activation mediated by mGluRs. This effect is thought to be important in facilitating sensory transmission to activate arousal and cognitive processes (McCormick and von Krosigk, 1992).

Activation of mGluRs can also produce inward currents separate from inhibition of potassium channels (Glaum and Miller, 1992; Mercuri *et al.*, 1993; Crépel *et al.*, 1994; Guérineau *et al.*, 1995). These inward currents vary in different types of cells and sometimes involves a Ca^{2+} -activated non-specific cation (CAN) current (Crépel *et al.*, 1994; Guérineau *et al.*, 1995; Congar *et al.*, 1997), or an activation of a Na^+/Ca^{2+} exchanger (Mercuri *et al.*, 1993; McBain *et al.*, 1994; Keele *et al.*, 1997). In the hippocampus the CAN current is mediated by Group I mGluRs because the selective agonist DHPG produces a current identical to that evoked by ACPD and this effect involves a Ca^{2+} -dependent and G-protein-dependent process (Congar *et al.*, 1997). In neurons of the basolateral amygdala, the metabotropic-activated Na^+/Ca^{2+} exchanger is elicited by DHPG, which may play a role in epilepsy, increasing transmission following kindling (Keele *et al.*, 1997).

Activation of Group I mGluRs can also modulate currents through N-type, L-type and other voltage-dependent calcium channels [Sayer *et al.* (1992); Sahara and Westbrook (1993); Swartz and Bean (1992); Swartz *et al.* (1993); for review see Stefani *et al.* (1996)]. Quisqualate inhibits N-type Ca^{2+} channels in hippocampus and cortex, where it is mediated by G-protein and does not involve protein kinases (Lester and Jahr, 1990; Sayer *et al.*, 1992; Swartz and Bean, 1992), but it facilitates L-type channel function in the granule cells of the cerebellum via a G-protein mechanism not sensitive to pertussis toxin (PTX) (Chavis *et al.*, 1995). In cerebellar granule cells, activation of mGluR₁ triggers a functional coupling between ryandine receptors and L-type Ca^{2+} channels that leads to facilitation of the L-type Ca^{2+} channel and could be

important in regulating a phenomenon of synaptic plasticity like long-term depression (LTD) (Chavis *et al.*, 1996). In fronto-parietal cortical neurons, inhibition of N-type Ca^{2+} channels produced by specific Groups I and II mGluRs agonists is reduced by MCPG, suggesting that both these mGluR groups participate in modulating Ca^{2+} channels in cortical neurons (Choi and Lovinger, 1996). However, a differential sensitivity to PTX can distinguish the two groups of mGluR. Inhibition of Ca^{2+} channels by Group II mGluRs seems to involve a PTX-sensitive G-protein whereas Group I mGluRs use both a PTX-sensitive and PTX-insensitive G-protein. These results suggest that mGluRs inhibiting Ca^{2+} channels in cortical neurons might be separated on the basis of the G protein involved (Choi and Lovinger, 1996). Moreover, Group I in contrast to Group II mGluRs can use several distinct signal transduction pathways to inhibit Ca^{2+} channels, both Ca^{2+} intracellular-independent and -dependent mechanisms (McCool *et al.*, 1998).

In the mGluR₁-rich cerebellum, ACPD increases excitation and firing rate of Purkinje cells both *in vivo* or in slices. MCPG and 4CPG both block the agonist-induced excitatory responses (Lingenhöhl *et al.*, 1993; Batchelor *et al.*, 1994). Two Group I mGluR antagonists, 4C3HPG and 4CPG, selectively depress ACPD-induced excitation of thalamic neurons in the anesthetized rat (Eaton *et al.*, 1993a; Salt and Eaton, 1995). ACPD also causes depolarization of neonatal rat motoneurons which is blocked by MCPG, 4C3HPG or 4CPG (Birse *et al.*, 1993), but not by 2S,1'S,2'S-2-methyl-2-(carboxycyclopropyl)-glycine (MCCG) or S-2-amino-2-methyl-4-phosphonobutanoate (MAP4), respectively, Groups II and III antagonists (Jane *et al.*, 1994).

Recent studies have shown that selective activation of Group I mGluRs with DHPG increases postsynaptic membrane excitability in hippocampus (Davies *et al.*, 1995), cortex (Mannaioni *et al.*, 1996; Libri *et al.*, 1997), striatum (Pisani *et al.*, 1997), amygdala (Keele *et al.*, 1997), subthalamic nucleus (Abbott *et al.*, 1997), hypothalamic supraoptic nucleus (Schrader and Tasker, 1997) and in motoneurons of the spinal cord (Ugolini *et al.*, 1997).

Although the profiles of agonist and antagonist pharmacology indicate that Group I mGluRs mediate postsynaptic depolarizations in a number of brain areas, the lack of subgroup-specific agents has prevented a determination of the actual subtype involved. Some indication of the roles of the two Group I subtypes comes from experiments where mice lacking specific mGluRs were generated. In CA1 neurons of mGluR₁-deficient mice, ACPD produced excitatory effects similar to normal (Aiba *et al.*, 1994a; Conquet *et al.*, 1994), whereas mGluR₅-deficient mice lacked the normal neuronal depolarization in response to ACPD (Lu *et al.*, 1997). Administration of mGluR₅ antisense deoxynucleotides injected into the hippocampal CA1 gave similar results (Dorri *et al.*, 1997). These results suggest that mGluR₅ mediates excitatory effects in pyramidal CA1 neurons where mGluR₁ is not expressed (Luján *et al.*, 1996), but they do not preclude a role for mGluR₁ in other regions. To test whether mGluR₁ would compensate for the absence of

mGluR₅ one should examine depolarization responses induced by mGluR agonists either in the mGluR₅-deficient mice or in animals injected with mGluR₅ antisense in brain areas where both receptors are present (e.g. dentate gyrus, hippocampus CA3).

Recent studies using cells expressing mGluR₁ or mGluR₅ only have found separate functions for the two Group I mGluR subtypes. While either receptor triggers the release of Ca²⁺ from intracellular stores through the IP₃ pathway, glutamate stimulation produces a single peak of intracellular Ca²⁺ mobilization in the cells transfected with mGluR₁, but Ca²⁺ oscillations in the mGluR₅ transfected cells. These oscillations are also seen in astrocytes expressing mGluR₅ and are PKC-dependent because they are blocked by the selective inhibitor H-7 (Kawabata *et al.*, 1996; Nakahara *et al.*, 1997; Nakanishi *et al.*, 1998). Interestingly, Ca²⁺ oscillations are found in CA1 hippocampal interneurons in response to glutamate stimulation and they are prevented completely by MCPG but only partially by co-administration of the iGluR antagonists AP5 and 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) (Carmant *et al.*, 1997).

4.2. Modulation of Synaptic Transmission

4.2.1. Presynaptic Inhibition

Synaptic transmission is influenced by mGluRs via modulation of postsynaptic receptors, as described above, but also by modulation of transmitter release from presynaptic terminals. Activation of presynaptic mGluRs cause a widespread reduction of glutamatergic transmission in the CNS. This action is determined almost exclusively by presynaptic Group II and Group III mGluRs [Baskys and Malenka (1991); Desai and Conn (1991); Pook *et al.* (1992); Burke and Hablitz (1994); Glaum and Miller (1994) for review]. Reduction of glutamatergic transmission attributed to Group I mGluRs has been observed only in the hippocampus, at the level of the synapses between Shaffer collaterals and CA1 pyramidal cells. In this region inhibition of EPSPs is induced by DHPG or quisqualate and is accompanied by an increase in paired-pulse facilitation, a common and reliable test of presynaptic mechanisms (Gereau and Conn, 1995b; Manzoni and Bockaert, 1995). mGluR₅ probably mediates this effect because it is not blocked by 4CPG (an mGluR₁ preferential antagonist) (Manzoni and Bockaert, 1995) and because there is immunochemical evidence for the presynaptic expression of mGluR₅ in CA1 neurons (Romano *et al.*, 1995).

Interestingly, a recent report demonstrated that the DHPG-induced inhibition of EPSPs in CA1 is facilitated by activation of NMDA receptors (Harvey *et al.*, 1996). According to these authors this mechanism in CA1 may have important implications for synaptic plasticity where both mGluRs and NMDA receptors are involved.

4.2.2. Modulation of GABA

A third function of mGluRs in the CNS is to regulate inhibitory synaptic transmission mediated by the amino acid neurotransmitter GABA. Activation of mGluRs reduces GABAergic synaptic transmission in the hippocampus (Liu *et al.*, 1993; Jouvenceau *et al.*, 1995), the striatum (Calabresi *et al.*, 1992; Stefani *et al.*, 1994), the thalamus (Salt and Eaton, 1995), the olfactory bulb (Hayashi *et al.*, 1993), and the nucleus of the tractus solitarius (Glaum and Miller, 1993). The identity of the mGluR Group responsible for this effect is not certain, but evidence for a presynaptic activation of Group II or Group III receptors is emerging.

In the hippocampus, GABAergic interneurons can be activated by mGluRs either pre- or postsynaptically (Desai *et al.*, 1994; McBain *et al.*, 1994; Jouvenceau *et al.*, 1995; Poncer *et al.*, 1995; Doherty and Dingledine, 1998). In the CA3 region activation of Group I mGluRs, presumably located on the somato-dendritic membrane of GABA interneurons, enhances excitability. In contrast, Group II mGluRs are located on inhibitory terminals and reduce GABA release (Poncer *et al.*, 1995). In the CA1 region, Group I mGluRs are involved in modulating transmission from GABAergic interneurons onto pyramidal cells. Analysis of spontaneous inhibitory postsynaptic currents (IPSCs) suggests that these receptors are localized presynaptically (Gereau and Conn, 1995b). Activation of Group I mGluRs on GABA inhibitory neurons in the hippocampus can contribute to the generation of hyperexcitability connected with epilepsy (Doherty and Dingledine, 1998) and may have an important role in synaptic plasticity, as shown in mice lacking mGluR₁ [Bordi *et al.* (1997) see later].

In the rat frontal cortex Group I mGluRs can enhance GABA-mediated synaptic inhibition through activation of inhibitory interneurons and, as in CA1, these receptors seem to be located presynaptically (Chu and Hablitz, 1998).

4.2.3. Modulation of Other Neurotransmitters

Recent work has focused on the interaction of mGluRs with other neurotransmitter systems. It is now becoming evident that mGluRs regulate different neurotransmitters in a number of brain structures.

Activation of rat dentate gyrus neurons with ACPD or the specific Group I mGluR agonist DHPG increases NPY mRNA levels in granule cells and interneurons and this effect is antagonized by MCPG and 4CPG (Schwarzer and Sperk, 1998). These results are important in light of the recent evidence of a possible role of NPY in epilepsy (Schwarzer *et al.*, 1998).

In the hippocampus DHPG is also able to attenuate the inhibitory effects of adenosine A1 receptor activation and this attenuation occurs via a PKC-dependent mechanism. This interaction may be relevant to the pathology that occurs after hypoxia, where adenosine is an endogenous protective substance (de Mendonça and Ribeiro, 1997; Budd and Nicholls, 1998).

In cultures of astrocytes, mGluR₅ is associated with the regulation of β -adrenergic receptor function. The agonist DHPG, but not agonists for Group II or III mGluRs, potentiates cyclic AMP accumulation induced by β -adrenergic stimulation. This effect is independent of intracellular Ca^{2+} or the PKC pathway (Balázs *et al.*, 1998), but can contribute to the opening of Ca^{2+} channels and modulate neuronal activity by a feedback process to neurons (Verkhatsky and Kettenmann, 1996; Balázs *et al.*, 1998).

Recent work showed that mGluRs modulate dopamine (DA) neurons in various ways. ACPD produces an increase of the spontaneous firing rate and a depolarization of rat DA mesencephalic cells (Mercuri *et al.*, 1993). This excitation is attributed in part to an inward current that is antagonized by the Group I mGluR antagonist 4C3HPG. In the ventral midbrain, ACPD and DHPG cause a decrease in the EPSP's amplitude and depolarization of DA neurons (Wigmore and Lacey, 1998). ACPD mediates excitation and inhibition of substantia nigra DA neurons as well, and this action is blocked by Group I mGluR antagonist 4CPG (Meltzer *et al.*, 1997). Moreover, electrical stimulation of ventral tegmental DA neurons evokes a slow excitatory postsynaptic current (EPSC) which is mediated by mGluRs, particularly by Group I mGluRs because it is blocked by MCPG (Shen and Johnson, 1997). In primary cultures of striatal neurons, Group I mGluR agonists potentiate the cAMP formation induced by activation of D_1 -like dopamine receptors. This potentiation is blocked by 4CPG and also by PKC inhibitors (Paolillo *et al.*, 1998).

A role of Group I mGluRs in modulating DA system function was shown also by the observation that intrastratial injection of ACPD or DHPG caused a contralateral rotational behavior, a response that is DA-dependent (Sacaan *et al.*, 1991, 1992; Kearney *et al.*, 1997). When infused into the nucleus accumbens, ACPD increases locomotor activity in rats and MCPG blocks this effect. Blockade of DA receptors with haloperidol reduces (in a dose-dependent manner) the ACPD-induced locomotor activation (Attarian and Amalric, 1997), suggesting that the increase in locomotor activity is dependent on DA. A recent study has shown that MCPG injected into the nucleus accumbens also interferes with amphetamine-induced locomotion, and suggested that the mGluRs located postsynaptically to DA terminals mediate this action (Kim and Vezina, 1998a).

All these results strongly suggest a possible involvement of Group I mGluRs in Parkinson's Disease or Huntington's Chorea by modulating the DA system [Sacaan *et al.* (1992); see later].

4.2.4. Modulation of Ionotropic GluRs

Activation of mGluRs has been shown to modulate *N*-methyl-D-aspartate (NMDA) and (*S*)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated membrane currents in a number of brain areas and cell types. Potentiation of NMDA responses by ACPD was reported in the CA1 region of the hippocampus

(Aniksztejn *et al.*, 1992; Harvey and Collingridge, 1993), cerebellum (Kinney and Slater, 1993), neocortex (Rahman and Neuman, 1996; Mannaioni *et al.*, 1996), visual cortex (Wang and Daw, 1996), striatum (Pisani *et al.*, 1997), and spinal cord (Cerne and Randic, 1992; Jones and Headley, 1995; Ugolini *et al.*, 1997). In some studies, however, ACPD attenuates NMDA responses (Colwell and Levine, 1994; Yu *et al.*, 1997) and this discrepancy can be explained by the NMDA receptor composition in different cell types (Shen *et al.*, 1995).

ACPD also potentiates AMPA responses in spinal cord (Cerne and Randic, 1992; Bleakman *et al.*, 1992; Jones and Headley, 1995; Bond and Lodge, 1995; Ugolini *et al.*, 1997) and visual cortex (Wang and Daw, 1996), but not in the hippocampus (Aniksztejn *et al.*, 1992; Harvey and Collingridge, 1993). Spinal cord depolarizations induced by kainate are increased by ACPD in some reports (Bleakman *et al.*, 1992; Jones and Headley, 1995) but not in others (Cerne and Randic, 1992; Ugolini *et al.*, 1997). Figure 1 illustrates the facilitation of NMDA or AMPA responses that is induced by ACPD in spinal cord motoneurons. The specific agonist DHPG produces effects similar to those obtained with ACPD in the hippocampus (Fitzjohn *et al.*, 1996), striatal slices (Pisani *et al.*, 1997) and spinal cord (Jones and Headley, 1995; Ugolini *et al.*, 1997).

The potentiation of excitatory responses is explained by an mGluR-mediated membrane depolarization that increases cell excitability (Jones and Headley, 1995; Bond and Lodge, 1995). Inhibition of K^+ currents (I_m or $I_{K \text{ leak}}$) are implicated in these mGluR-induced depolarizations (Charpak *et al.*, 1990; Gerber and Gähwiler, 1994), resulting in an increase in cell input resistance.

PKC, which is triggered by activation of Group I mGluRs, has been proposed to mediate the potentiation of iGluR responses. In oocytes a specific PKC antagonist blocks the mGluR-induced enhancement of the NMDA response (Kelso *et al.*, 1992). In the CA1 region of the hippocampus, potentiation of the NMDA effect induced by ACPD was blocked by PKC antagonists in one study (Aniksztejn *et al.*, 1992), but not in another (Harvey and Collingridge, 1993). In the striatum, the PKC blockers staurosporine or calphostin C are effective in preventing DHPG-mediated potentiation of the NMDA-induced depolarization and the PKC activator phorbol-12,13-diacetate (PDAC) mimicks the enhancement of the NMDA responses (Pisani *et al.*, 1997). In spinal cord motoneurons, the potentiation of responses elicited by both NMDA and AMPA is blocked by the two PKC blockers, staurosporine or chelerythrine chloride [Ugolini *et al.* (1997) see Fig. 1], suggesting the involvement of a PKC-dependent mechanism in the mGluR-induced potentiation in the spinal cord.

PKC phosphorylates NMDA and AMPA receptors (Roche *et al.*, 1994). The activation by mGluRs of PKC might therefore modulate the phosphorylation state of these two ionotropic receptors, resulting in a potentiation of the response [see Michaelis (1998)]. This mechanism could also explain how mGluR potentiation of NMDA and AMPA re-

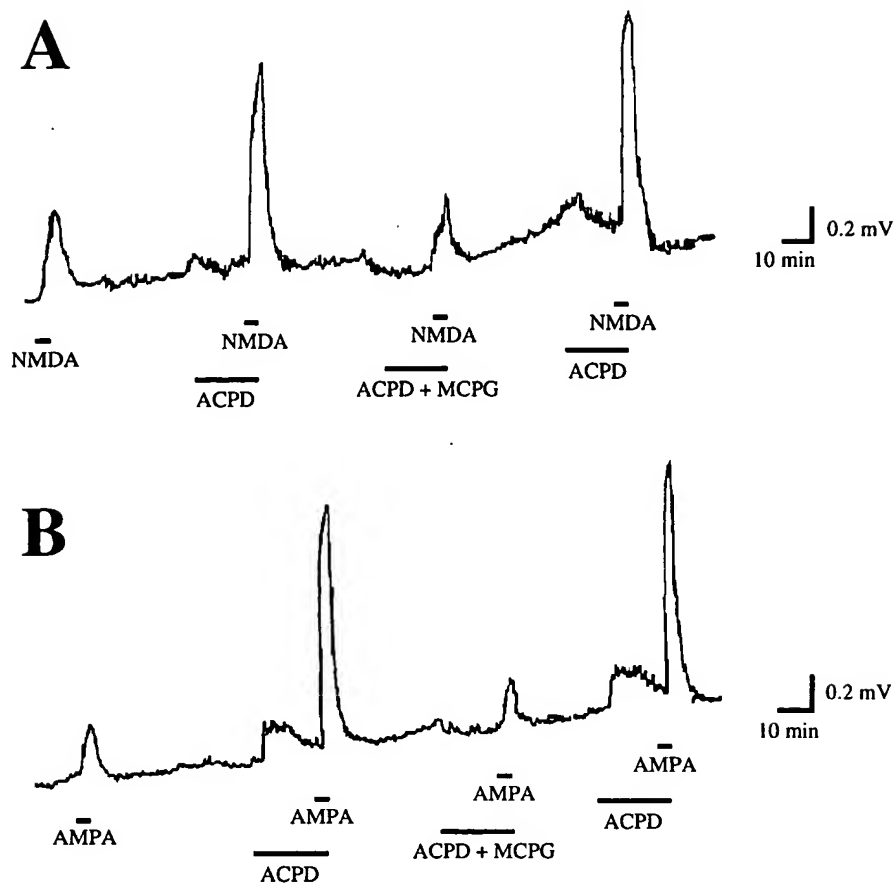


Fig. 1. The mGluR agonist ACPD facilitates the depolarization of motoneurons induced by NMDA or AMPA. This facilitation is blocked by the Group I mGluR antagonist MCPG. Top trace (A) is a representative recording from one experiment in which NMDA ($10 \mu\text{M}$, 4 min) potentiation caused by perfusion of ACPD ($10 \mu\text{M}$, 20 min) is blocked when ACPD is perfused together with MCPG ($500 \mu\text{M}$). In the bottom trace (B) an individual trace is taken from one experiment in which MCPG ($500 \mu\text{M}$) antagonizes the ACPD-induced potentiation of AMPA ($0.1 \mu\text{M}$, 4 min) responses [reproduced from Ugolini *et al.* (1997)].

sponses could be involved (or participate in) some forms of plasticity like long-term potentiation (LTP) or nociception [Harvey and Collingridge (1993); Corsi *et al.* (1997); see below].

The specific mGluR₅ agonist CHPG is also able to potentiate NMDA responses in the hippocampus (Doherty *et al.*, 1997) and NMDA or AMPA responses in spinal cord motoneurons (Ugolini and Bordi, unpublished observations), suggesting a role for mGluR₅ in this action either alone or in combination with mGluR₁ (Fig. 2). In the spinal cord, however, there is no evidence for an involvement of PKC, in contrast to what it is seen when the NMDA or AMPA responses are potentiated by ACPD. Both mGluR₅ and mGluR₁ may thus act to enhance iGluR responses but the two types of mGluRs may have different intracellular mechanisms of action. Different functions for the two types of Group I mGluRs has also been suggested by recent studies with cells transfected with mGluR₁ and mGluR₅ [see Nakanishi *et al.* (1998)].

4.3. Long-Term Regulation of Synaptic Efficacy

Group I mGluRs have been implicated in different forms of synaptic plasticity, such as LTP and LTD, and in memory formation (Bliss and Collingridge, 1993; Riedel and Reymann, 1996). LTP and LTD are persistent changes in synaptic efficacy induced by tetanic afferent stimulation and are considered models of the cellular mechanisms underlying learning and memory (Bliss and Lomo, 1973; Bear and Malenka, 1994).

4.3.1. LTP and Memory

Facilitating effects of the mGluR agonist ACPD in LTP potentiation have been shown in hippocampal slices. Pre-treatment with the mGluR agonist causes increased LTP following tetanus compared to untreated controls (Otani and Ben-Ari, 1991; McGuinness *et al.*, 1991). ACPD can also induce LTP in absence of electrical stimulation. This LTP induction depends on an intact connection between

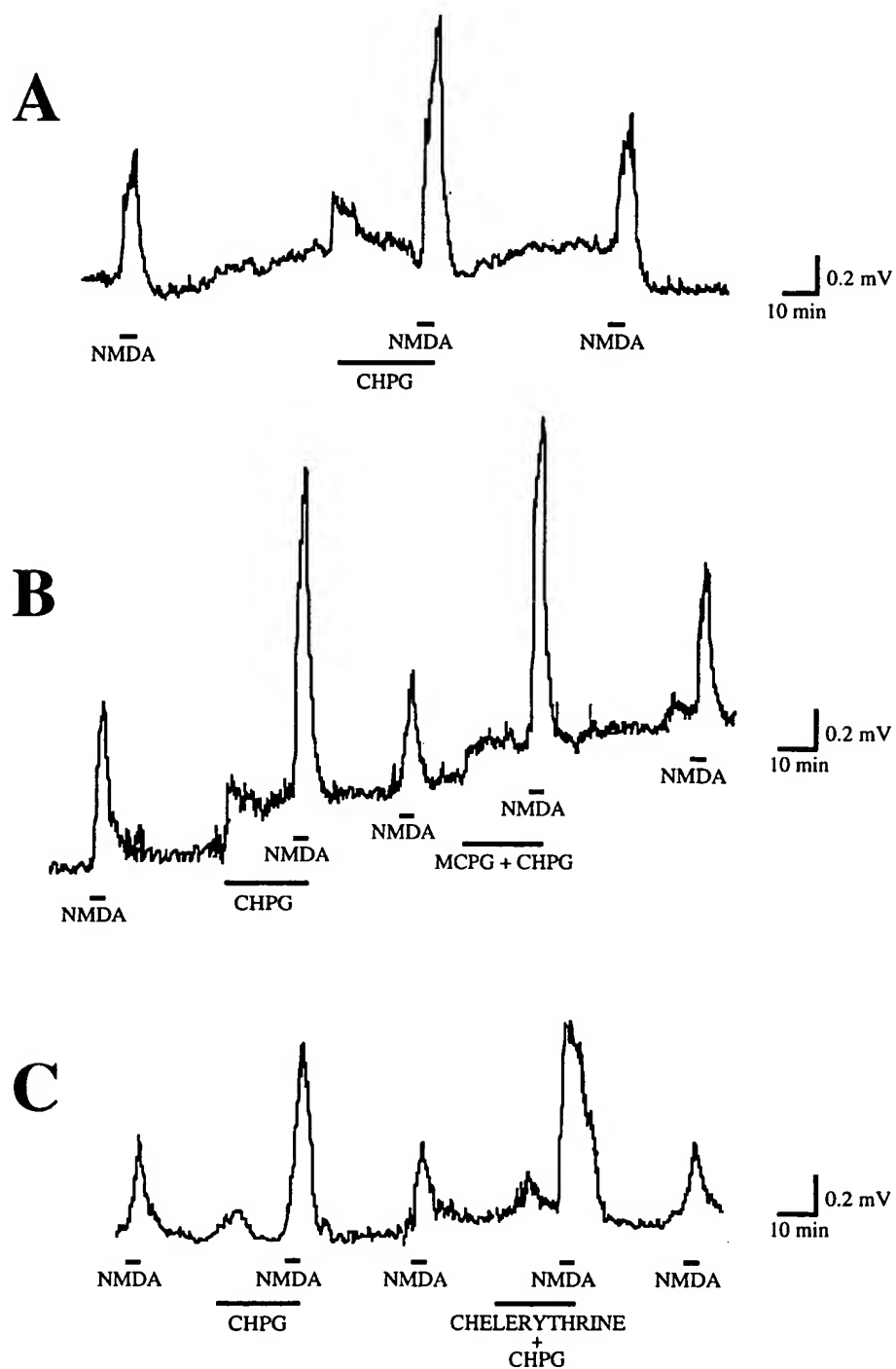


Fig. 2. The mGluR₅ agonist CHPG enhances the depolarization of motoneurons induced by NMDA. This facilitation is neither blocked by the Group I mGluR antagonist MCPG nor by the PKC inhibitor chelerythrine chloride. Each trace is taken from an individual experiment. Periods of drug perfusion are indicated by bars for NMDA (10 μ M, 4 min) and CHPG (1 mM, 20 min). Chelerythrine chloride (3 μ M) or MCPG (1 mM) are co-applied with CHPG.

the CA3 and CA1 regions of the hippocampus (Bortolotto and Collingridge, 1993, 1995; Breakwell *et al.*, 1996). Since ACPD exerts its facilitating action through a PKC-dependent pathway (Anwyl, 1991; Otani *et al.*, 1993), a mechanism proposed to explain the involvement of mGluRs in LTP is by modulation of NMDA receptors via PKC. This view holds that NMDA is sufficient to generate LTP (Ben-Ari and Aniksztejn, 1995). A different view suggests that mGluRs trigger directly LTP via activation of PKC in parallel with induction involving NMDA receptors (Watkins and Collingridge, 1994).

It has been difficult to distinguish between these hypotheses using the available mGluR antagonists. MCPG can inhibit both tetanus-induced and ACPD-induced LTP in the hippocampus CA1 (Bashir *et al.*, 1993). However, MCPG blocked LTP only in 'naïve slices', but not in slices that had already been stimulated tetanically (Bortolotto *et al.*, 1994). The authors proposed that Group I mGluRs activate a molecular switch that, once stimulated, stays on for a long time and need not be activated again. This switch is PKC-mediated, since its activation is blocked by PKC inhibitors, but it does not require co-activation of NMDA receptors, and can be reset by low frequency stimulation that depotentiates LTP and re-establishes the need for mGluR-activation for LTP in non-naïve slices (Bortolotto *et al.*, 1994).

The effects of MCPG on LTP have been quite variable, however. While some reports confirmed the original blocking effect exerted by MCPG in the hippocampus (Sergueeva *et al.*, 1993; O'Connor *et al.*, 1994; Little *et al.*, 1995) or in the medial frontal cortex (Vickery *et al.*, 1997), other studies failed to find any effect of MCPG in inhibiting tetanus-induced or ACPD-induced LTP in the hippocampal (Chinestra *et al.*, 1993; Manzoni *et al.*, 1994; Selig *et al.*, 1995; Thomas and O'Dell, 1995) or in visual cortical slices (Hensch and Stryker, 1996; Huber *et al.*, 1998). Part of these conflicting results have been explained by the different conditions in which the slice is placed in the recording chamber (Bortolotto *et al.*, 1995; Ben-Ari and Aniksztejn, 1995; Breakwell *et al.*, 1996) or by the different developmental stage of the animals. Izumi and Zorumski (1994) found that MCPG given 5 minutes after tetanization was effective in inhibiting LTP in hippocampal slices taken from PND15 or 30 but not from PND60 rats.

Another factor that may explain different findings is the tetanization strength and the resulting intracellular response. Since it has been proposed that intracellular Ca^{2+} concentration is a critical factor for LTP induction (Malenka *et al.*, 1988, 1992), a recent study using Ca^{2+} imaging analysis suggests that Group I mGluRs involvement in LTP is confined to certain types of potentiation, which are induced by weak tetanization and require the release of Ca^{2+} from intracellular stores. The stronger the potentiation, the less are the mGluR antagonists able to inhibit LTP induction (Wang *et al.*, 1995; Wilsch *et al.*, 1998).

In adult rats, MCPG attenuates LTP in hippocampus dentate gyrus in anesthetized animals in one study (Richter-Levin *et al.*, 1994), but is completely

ineffective in other studies (Bordi and Ugolini, 1995; Martin and Morris, 1997). Since the mGluR antagonist readily blocks LTP induction in freely behaving animals (Riedel *et al.*, 1994, 1995; Riedel and Reymann, 1996), it is possible that the state of anesthesia may interact with the drug action or that the weaker tetanization parameters necessary to induce a stable LTP in freely-moving rats may unmask the role of mGluRs in LTP (Martin and Morris, 1997).

Studies of mice lacking mGluR₁ confirm the involvement of Group I mGluRs in LTP induction, although different results have been reported. In one study mGluR₁ $-/-$ mice had normal LTP in the CA1 or dentate gyrus areas, both NMDA receptor-dependent pathways, but reduced LTP in the mossy fiber CA3 area, which involves an NMDA receptor-independent pathway (Conquet *et al.*, 1994). The opposite result was found in a study using a different set of mGluR₁ knock-out mice (Aiba *et al.*, 1994a; Hsia *et al.*, 1995).

The question of LTP in mGluR₁-deficient animals was recently addressed by measuring LTP in the intact hippocampus of anesthetized mice. In these animals, LTP was reduced in the perforant path-dentate gyrus pathway (Bordi, 1996). A possible explanation of this result is that dentate gyrus neurons receive more inhibitory synaptic drive *in vivo* than in slice preparation where many inhibitory axon collaterals are lost (Buckmaster and Schwartzkroin, 1995). Decreasing the level of feedback inhibition by activation of GABA_B receptors on GABA interneurons made it possible to induce normal LTP in mGluR₁ $-/-$ mice [Bordi *et al.*, 1997; Fig. 3(A)]. A possible scenario is depicted in Fig. 3(B). According to this view glutamate controls GABA inhibition via mGluR₁ situated postsynaptically on the interneuron. mGluR₁ would thus have an indirect role in synaptic plasticity by regulating GABA inhibition.

Interestingly, a very similar mechanism has been described for a form of synaptic modulation exerted by mGluR₂ in the olfactory bulb. Here, activation of the mGluR₂-rich granule cells suppresses inhibitory GABA transmission to mitral cells allowing the formation of a specific olfactory memory (Hayashi *et al.*, 1993; Kaba *et al.*, 1994).

Mice lacking mGluR₅ show reduced LTP in the regions of the hippocampus that are known to be NMDA receptor-dependent, but they have a normal LTP in the mossy fiber synapses of the CA3 region (Lu *et al.*, 1997). Thus, mGluR₅ plays an important regulatory role in LTP in NMDA-dependent pathways in the hippocampus.

Treatments that interfere with LTP are usually also studied in cognitive tests (Morris *et al.*, 1986). To investigate whether reduced LTP resulted in an impairment in learning and memory, mGluR₅ mutant mice were tested in two different spatial learning tasks, both known to depend on an intact hippocampus, the Morris water maze (Morris, 1984) and the contextual fear conditioning test (Phillips and LeDoux, 1992). In both tasks, the performance of mGluR₅-deficient mice was significantly different than that of their wild-type littermates (Lu *et al.*, 1997). Interestingly, mGluR₁ mutant mice were also

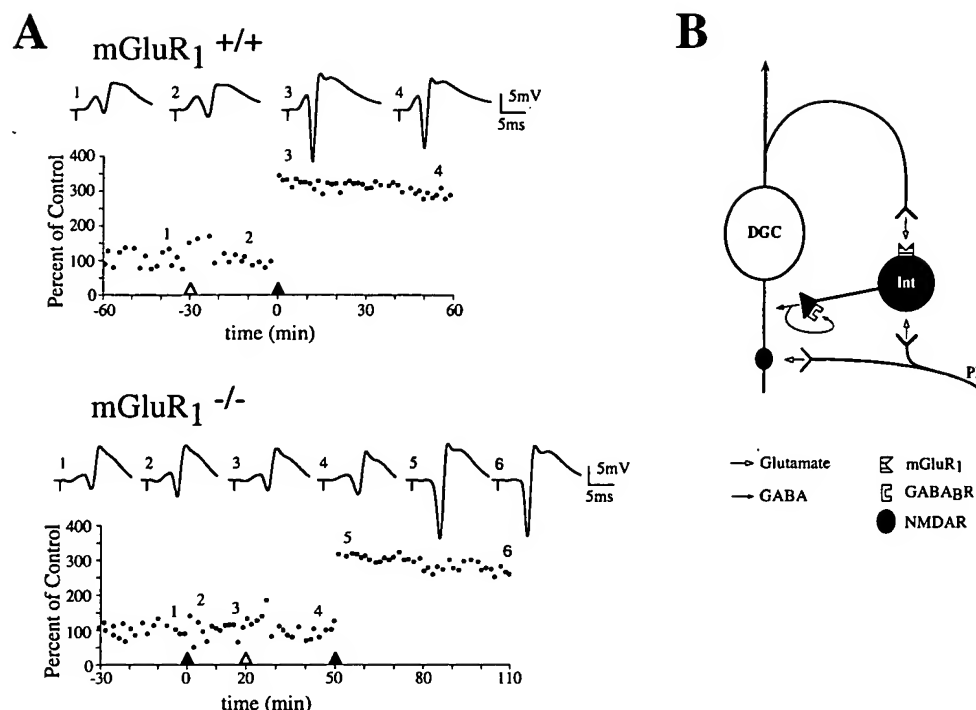


Fig. 3. (A) Effects of mGluR₁ +/+ mice and mGluR₁ -/- on perforant path-dentate gyrus LTP. Consistent LTP was induced in wild-type animals whereas mGluR₁ -/- animals showed a reduced LTP of population spike amplitude compared to control. Baclofen injection did not modify the ability to evoke LTP in wild-type animals, but allowed a normal induction of LTP in mutant -/- mice. Top traces are representative EPSPs taken from one experiment each at the time indicated on the graph. Each point is the average of three evoked potentials. ▲, When the tetanus was induced (three trains 10 sec apart, 400 Hz, eight 0.4 msec impulses in each train). △, Start of the baclofen injection (10 mg kg⁻¹ ml⁻¹, i.p.). (B) Schematic diagram of the basic dentate gyrus circuit with proposed localization of mGluR₁. The GABA-releasing interneuron (Int) can be activated by synapses from the glutamatergic perforant path (PP) or via a recurrent collateral from the dentate gyrus granule cell (DGC). When GABA is released from the interneuron it acts on presynaptic GABA_A receptors [reproduced from Bordi *et al.* (1997)].

impaired in the same behavioral tasks (Conquet *et al.*, 1994; Aiba *et al.*, 1994a). Taken together these results strongly implicate Group I mGluRs in learning and memory function. However, since the mice are congenitally lacking mGluR₁ or mGluR₅, it is possible that these two receptors are only required during development to establish a plastic state, and may not be needed for the expression of learning and memory *per se*.

This argument leads to a review of the behavioral studies that tested animals in learning and memory tasks under pharmacological treatment to see if Group I mGluR antagonists also interfere with learning and memory. In fact, more consistent results of the effects of MCPG on behavior were obtained than on tests of LTP. Using different behavioral paradigms, a number of laboratories showed an impairment of the learning performance when MCPG was injected intraventricularly in rats. These include inhibition of water maze learning (Richter-Levin *et al.*, 1994; Bordi *et al.*, 1996) and of Y-maze with footshock reinforcement (Riedel *et al.*, 1994;

Balschun and Wetzel, 1998). Retention deficits have also been reported in passive avoidance learning in young chicks [Holscher (1994); Rickard and Ng (1995); see Riedel (1996) for review]. Using a more potent Group I antagonist, AIDA, Nielsen *et al.* (1997) found blockade of hippocampus-dependent contextual fear conditioning, but not hippocampus-independent cue conditioning. In contrast, context-dependent fear conditioning was not altered by MCPG (Bordi *et al.*, 1996). Similarly, intra-hippocampal injections of MCPG did not affect working memory in rats, but AIDA dose-dependently increased the number of errors in the three panel runway setup (Ohno and Watanabe, 1996, 1998). Co-application of MCPG and a NMDA receptor antagonist aggravates impairment in the working memory task, while infusion of a partial agonist at the glycine site, D-cycloserine, reduced the errors induced by AIDA. These findings suggest an interactive regulation of memory processes by mGluR- and NMDA receptor-mediated mechanisms (Ohno and Watanabe, 1996, 1998).

4.3.2. LTD

Together with LTP, LTD in the cerebellum or in the hippocampus is an important example of cellular mechanism of long-lasting synaptic plasticity.

There is clear pharmacological evidence that Group I mGluRs are involved in the induction of LTD at parallel fiber-Purkinje cell synapses in the cerebellum (Daniel *et al.*, 1992; Linden and Connor, 1993; Hartell, 1994). The antagonist MCPG also blocks the induction of homosynaptic LTD in the hippocampus (Bolshakov and Siegelbaum, 1994; O'Mara *et al.*, 1995) but does not always do so (Selig *et al.*, 1995; Oliet *et al.*, 1997). MCPG is effective in blocking LTD induced by low-frequency stimulation in the visual cortex in some studies (Kato, 1993; Haruta *et al.*, 1994; Hensch and Stryker, 1996), but a recent report fails to reproduce the same results (Huber *et al.*, 1998). A possible explanation of the discrepancies found both in the hippocampus and in the visual cortex is that there are two forms of LTD, one presynaptic and MCPG-sensitive, the other postsynaptic and NMDA receptor-dependent (Oliet *et al.*, 1997; Nicoll *et al.*, 1998).

Activation of Group I mGluRs with the selective agonist DHPG or with the mGluR₅ specific agonist CHPG induces LTD in CA1 hippocampus slices in Mg²⁺-free medium or with GABA-mediated inhibition blocked. Reduction of DHPG-induced LTD in Mg²⁺-free medium is achieved by MCPG or by application of the NMDA receptor antagonist (R)-2-amino-5-phosphonopentanoate (AP5) (Palmer *et al.*, 1997). Application of ACPD in CA1 slices of immature rats causes LTD and this effect is also blocked by NMDA receptor antagonists (Overstreet *et al.*, 1997). Together, these results indicate that mGluRs and NMDA receptor-dependent forms of LTD may not be completely independent processes (Palmer *et al.*, 1997).

A study using freely-moving animals found that MCPG and 4CPG inhibited LTD induction in the hippocampus while Group II mGluR antagonists modulate LTD maintenance (Manahan-Vaughan, 1997).

Mice lacking mGluR₁ show reduced LTD in cerebellar Purkinje cells (Aiba *et al.*, 1994b; Conquet *et al.*, 1994). This electrophysiological phenomenon is accompanied by impaired eyeblink conditioning (Aiba *et al.*, 1994b) and severe motor coordination deficits, probably the result of cerebellar dysfunction. There are not, however, gross abnormalities of the basic bioelectric properties or of excitatory and inhibitory synaptic responses in these cells (Conquet *et al.*, 1994). The results from mGluR₁-deficient mice imply a role for these receptors in cerebellar LTD, as it was suggested by pharmacological experiments (Daniel *et al.*, 1992; Linden and Connor, 1993; Hartell, 1994) or by studies in which antibodies directed against the N-terminal extracellular segments of mGluR₁ blocked LTD induction in cultured Purkinje neurons (Shigemoto *et al.*, 1994). The other Group I mGluR, mGluR₅, does not compensate for the lack of LTD response in mGluR₁-deficient mice. In addition, mice lacking mGluR₅ do not exhibit motor deficits and they have a normal LTD (Lu *et al.*, 1997).

5. GROUP I mGLURs IN PATHOLOGICAL CONDITIONS

Glutamate is a major neurotransmitter that mediates synaptic excitation at the majority of CNS synapses and it is therefore involved in many important brain functions. Because of its widespread presence, glutamate has been implicated in a variety of CNS disorders. Overstimulation of glutamate receptors results in the death of neurons, a phenomenon called excitotoxicity that has been linked to several pathological states in the CNS, such as brain ischemia, hypoxia and traumatic brain injury. If this excessive glutamatergic activation is blocked by competitive or non-competitive glutamate receptor antagonists it is possible to reduce the hypoxic-ischemic brain damage (Rothman and Olney, 1986).

Excitotoxicity has also been related to epilepsy and may have a role in the pathogenesis of chronic neurodegenerative disorders such as Huntington's disease or Alzheimer's disease (Greenamyre and Young, 1989; Albin and Greenamyre, 1992). Whether the neurotoxic actions of glutamate are the primary cause of these diseases or a final common pathway to neuronal death in many neurological disorders is still a matter of numerous debates (Greenamyre and Porter, 1994; Kornhuber and Wiltfang, 1998). However, pharmacological agents that reduce glutamate receptor activation might provide a valuable therapeutic approach for neuroprotection of acute and chronic neurodegenerative disorders.

Modifying glutamatergic transmission with drugs acting selectively at Group I mGluRs can have important potential benefits. By developing drugs capable of inhibiting discrete populations of glutamatergic receptors it may be possible to bypass the profound side-effects that have limited the clinical use of iGluR antagonists (Olney *et al.*, 1989; Olney, 1994). Moreover, the ability of mGluRs to modulate the response of other neurotransmitters may also be crucial in the management of neurological and psychiatric illnesses.

In the following section the involvement of mGluRs belonging to Group I in pathophysiological processes will be reviewed.

5.1. Role of Group I mGluRs in Nociception

There is considerable evidence for the involvement of the excitatory amino acids, glutamate and aspartate, in transmission of both acute and chronic pain (Besson and Chaouch, 1987; Willis and Westlund, 1997; Dickenson *et al.*, 1997). A large number of peripheral sensory fibers contain glutamate, including C-fibers, and *ca* 80% of substance P-containing fibers also contain glutamate (Battaglia and Rustioni, 1988).

In the spinal cord, the response to brief acute mechanical or thermal stimuli appears to involve AMPA receptor activation (Aanonsen *et al.*, 1990). When the stimulus is maintained and/or its frequency or intensity is increased, however, NMDA receptors become activated, resulting in a potentiation of the response that underlies many forms of central sensitization (Dickenson, 1995). Sensitization

is an increase in response to stimulation and has been studied experimentally in the spinal cord using the 'wind-up' phenomenon (Woolf, 1983; Woolf and Wall, 1986), an increase in the number of spikes generated by a neuron after each successive stimulus during a pulse train [see Baranauskas and Nistri (1998) for review]. The wind-up is suggested to be a central mechanism for hyperalgesia (Mendell and Wall, 1965). Glutamate or NMDA reproduce the phenomenon of wind-up (King *et al.*, 1988), whereas NMDA antagonists can prevent it (Dickenson and Sullivan, 1987; Davies and Lodge, 1987).

Recent evidence suggests the involvement of mGluRs in nociceptive transmission, in accord with immunohistochemical distribution studies showing the presence of Group I mGluRs in layers I and II of the dorsal horn (Vidnyánszky *et al.*, 1994; Valerio *et al.*, 1997b). The potential importance of these receptors was suggested by their role in another sensitization phenomenon, LTP in the hippocampus, and by the facilitation induced by ACPD of AMPA and NMDA responses in dorsal horn neurons of the spinal cord [Bleakman *et al.* (1992); Cerne and Randic (1992); see Fig. 1].

Like NMDA receptor antagonists, the mGluR antagonist MCPG shows a significant inhibition of wind-up (Boxall *et al.*, 1996). Moreover, ACPD enhances wind-up of dorsal horn neurons and this effect is blocked by 4C3HPG, confirming the involvement of Group I mGluRs (Budai and Larson, 1998). Single shock electrical stimulation of the spinal cord dorsal root, sufficient to recruit both A- and C-fibers, is also used to study *in vitro* the response to acute noxious stimulation (Thompson *et al.*, 1992). Unlike the NMDA receptor antagonist AP5, MCPG is able to attenuate the late prolonged component of ventral root potentials (Boxall *et al.*, 1996). The late phase, mostly attributed to C-fiber activation, is defined as 'peptidergic' because it is also affected by neurokinin receptor antagonists (Thompson *et al.*, 1992, 1993) and might therefore involve second messenger systems. The early, monosynaptic component of the response, dependent on AMPA and kainate receptors, is not affected by MCPG or 4CPG (Boxall *et al.*, 1996; Corsi *et al.*, 1997). Figure 4 depicts the effects of MCPG on ventral root potentials after single shock stimulus of the dorsal root and after wind-up evoked by repetitive stimulation.

Excitation of dorsal horn cells induced by the Group I selective agonist DHPG is much greater than that evoked by Groups II and III agonists, suggesting a functional role for Group I mGluRs in this region (Young *et al.*, 1997). Activation of dorsal horn neurons is also produced by cutaneous application of the C-fiber chemical irritant mustard oil that brings about central sensitization of these neurons to afferent input, a process analogous to wind-up. The sensitization induced by mustard oil is inhibited by ionophoretic application of the selective Group I antagonist 4C3HPG (Young *et al.*, 1994, 1995), consistent with the observation that mGluRs are required in the inflammation induced by carrageenan (Neugebauer *et al.*, 1994).

These results suggest that, in addition to NMDA receptors, Group I mGluRs are important in the

generation of the spinal cord nociceptive response. Since PKC inhibitors, as well as Ca^{2+} /calmodulin-dependent kinase II inhibitors, also attenuate nociceptive responses, it has been suggested that PKC and possibly Ca^{2+} /calmodulin kinase II play a role in sensitized nociception in the dorsal horn and that Group I mGluRs may be the synaptic mediators involved in triggering these signal transduction pathways. The activation of PKC by mGluRs could play this role via NMDA phosphorylation and enhancement (Young *et al.*, 1997).

Responses of thalamic neurons to noxious thermal somatosensory stimuli are also reduced by local ionophoretic application of Group I mGluR antagonists (Eaton *et al.*, 1993a,b). The effect is selective in that non-noxious vibrissal stimulation is not antagonized (Eaton *et al.*, 1993b). A recent study confirmed these findings using the selective mGluR₁ antagonist LY367385, demonstrating for the first time the specific contribution of this subclass of receptors in nociceptive responses (Salt and Turner, 1998). This result is in good agreement with the evidence of the prominent expression of mGluR₁ in the thalamus (Masu *et al.*, 1991; Shigemoto *et al.*, 1992; Fotuhi *et al.*, 1993).

Interestingly, as in the thalamus, synaptic responses evoked by innocuous vibrissal stimuli are not affected by MCPG in the rat somatosensory cortex, suggesting that Group I mGluRs in the cortex are also not involved in simple sensory transmission of mechanical stimuli (Cahusac, 1994). Instead, the action of mGluR antagonists seems to be selective for noxious stimuli.

Behavioral studies using the hot-plate model to study the nociceptive reflex show that 4CPG and MCPG injected intracerebroventricularly in mice induce a dose-related increase in paw-licking latency, although NMDA receptor antagonists seem to be more efficacious (Corsi *et al.*, 1997). Similar results were achieved using the more potent mGluR antagonist AIDA (Moroni *et al.*, 1997). Subcutaneous injections of formalin to the plantar surface of the rat hindpaw is a widely used animal model of persistent pain (Coderre *et al.*, 1990). The formalin test produces two phases: an immediate acute nociceptive phase up to 5 min post-injection followed by a prolonged tonic nociceptive response lasting 20–60 min. NMDA receptor antagonists reduce this late phase of nociceptive responses (Coderre and Melzack, 1992; Yamamoto and Yaksh, 1992). Similarly, rats pretreated with the mGluR antagonists 4CPG or 4C3HPG injected intrathecally have reduced nociceptive scores in the second phase of the formalin test, while the agonists DHPG or ACPD enhance these nociceptive responses in a dose-dependent manner (Fisher and Coderre, 1996a). If the antagonists 4C3HPG or MCPG are injected prior to the agonist, the enhancement of formalin-induced nociception is attenuated. Interestingly, the same blocking effect of mGluR-induced enhancement of formalin response can be achieved with the NMDA antagonist AP5, demonstrating the interaction between mGluRs and NMDA in this type of sustained pain (Fisher and Coderre, 1996a).

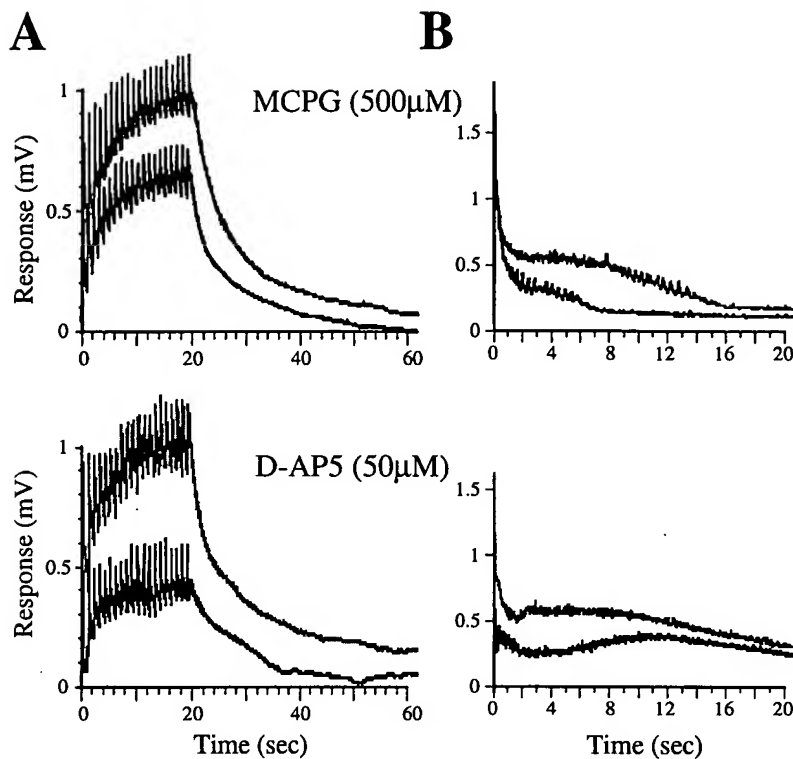


Fig. 4. Effects of D-AP5 (A) or MCPG (B) on high-intensity, single shock electrical stimulation (50 mV, 1 msec) of the dorsal root, and cumulative depolarization ('wind-up') of the ventral root evoked by 20 sec of 1 Hz repetitive electrical stimulation of the L_4/L_5 dorsal root. Traces represent different experiments. D-AP5 (50 μ M) or MCPG (500 μ M) are perfused for 20 min. They are able to reduce the amplitude of the wind-up or the amplitude of the late phase of the single shock evoked by dorsal root stimulation. Control traces for each experiment are depicted in gray. [reproduced from Corsi *et al.* (1997)].

Injection of DHPG or ACPD induces spontaneous nociceptive behavior which is blocked by Group I mGluR antagonists (Fisher and Coderre, 1996b). To investigate the relative contribution of mGluR1 or mGluR5 in the DHPG-induced responses, antibodies selective for the two receptor subtypes were injected intrathecally in rats. Both antibodies reduced the DHPG-induced nociceptive behavior (Fundytus *et al.*, 1998). Furthermore, both antibodies significantly attenuated hypersensitivity following chronic constriction injury (CCI) of the sciatic nerve but neither was effective in reducing nociceptive responses to acute heat or chemical stimuli (Fundytus *et al.*, 1998), supporting the notion that Group I mGluRs are primarily important in chronic, not acute, pain (Young *et al.*, 1997). Similar results were found using the CCI model with the Group I mGluR antagonist 4CPG used to attenuate chronic nociceptive behaviors (Fisher *et al.*, 1998).

Morphine is commonly used for the management of pain, although in a limited manner due to the development of tolerance and dependence after chronic use. Recent work has demonstrated that NMDA receptor antagonists attenuate the development of tolerance and dependence if co-administered

with morphine (Trujillo and Akil, 1991). A study by Fundytus and Coderre (1994) has shown that mGluRs activation, too, is involved in the development of opioid dependence with chronic morphine use, possibly through changes in intracellular messengers such as PI hydrolysis and DAG. Chronic antagonism of Group I mGluRs may act to compensate the increase in PI hydrolysis that is elevated by activation of μ -opioids particularly during withdrawal (Fundytus and Coderre, 1994; Fundytus *et al.*, 1997). Thus, treatments with Group I mGluRs, as well as with agonists of the other mGluR groups (Fundytus and Coderre, 1997), can be very effective in decreasing the incidence of opioid dependence.

In summary, blockade of Group I mGluRs, possibly both mGluR1 and mGluR5, may be a promising new route for treatment of pain, particularly chronic pain disorders (Young *et al.*, 1994, 1997; Neugebauer *et al.*, 1994; Fisher *et al.*, 1998), with minimal effects on normal sensory perception.

5.2. Role of Group I mGluRs in Epilepsy

Glutamate plays a crucial role in epileptogenesis. Overstimulation of iGluRs leads to seizures and excitotoxic injury throughout the CNS and in par-

ticular in the hippocampus, a vulnerable region to injury after seizures (Meldrum, 1994; Bradford, 1995). Glutamate antagonists selective for either NMDA or non-NMDA receptors are potent anticonvulsants in several different models of epilepsy (Patel *et al.*, 1990; Chapman *et al.*, 1991).

The mGluRs are currently under intense investigation to understand their role in epileptogenesis. Early reports showed that ACPD injected in rats or mice induces limbic seizures followed by selective neuronal degeneration (Sacaan and Schoepp, 1992; McDonald *et al.*, 1993; Tizzano *et al.*, 1993). Later studies extended these findings to the specific Group I agonist DHPG. DHPG-induced limbic seizures in mice (Tizzano *et al.*, 1995) and rats (Camón *et al.*, 1998) are not prevented by NMDA or AMPA/kainate receptor antagonists (Tizzano *et al.*, 1995). Both ACPD and DHPG have been suggested to exert their action by a common mechanism, most likely involving Ca^{2+} mobilization subsequent to PI-linked mGluR activation. L-2-amino-3-phosphonopropanoic acid (L-AP3) and dantrolene, inhibitors of mGluR-mediated intracellular calcium mobilization, prevent or attenuate the ACPD or DHPG-induced seizures in mice (Tizzano *et al.*, 1993, 1995).

In the audiogenic-induced convulsion model, the Group I mGluR antagonist 4C3HPG has an anticonvulsant action in mice (Thomsen *et al.*, 1994; Dalby and Thomsen, 1996). Similar suppression effects by 4C3HPG are found in rats that are genetically prone to epilepsy (Tang *et al.*, 1997). Although 4C3HPG is also an agonist for Group II mGluRs, various types of evidence, including the failure by the selective Group II agonist L-CGG-I to induce seizures, suggest that the acute convulsant action is predominantly mediated by antagonism of Group I receptors (Tang *et al.*, 1997).

Recordings of epileptic activities made *in vitro* in hippocampal or neocortical slices confirm the contribution of Group I mGluRs in the production of seizure discharges. Spontaneous epileptiform bursts in guinea pig hippocampal neurons elicited by exposure to the GABA receptor antagonist picrotoxin can be blocked by MCPG (Merlin *et al.*, 1995) or transformed into persistent prolonged discharges by the agonist DHPG (Merlin and Wong, 1997). Group I mGluRs may have a role in initiating the epileptogenesis process, which also requires active protein synthesis (Merlin *et al.*, 1998). In rat neocortical neurons, the GABA antagonist bicuculline induces epileptiform discharges that are suppressed by MCPG, although this antagonist fails to attenuate the ACPD-induced increase of epileptiform activity (Burke and Hablitz, 1995).

Exposure to 4-aminopyridine (4-AP), a blocker of voltage-gated K^+ channels, results in the generation of spontaneous discharges in brain slices that resembles interictal spiking and ictal epileptiform bursts (Rutecki *et al.*, 1987). MCPG selectively prevents the development of these events in the basolateral amygdala, without affecting the maintenance of the ictal discharges or the interictal spiking (Arvanov *et al.*, 1995). The mGluRs appear to be important in the transition from normal neuronal functioning to epileptiform bursting. Whether this effect is mediated by Group I or Group II mGluRs

is not yet clear, because MCPG is an antagonist for both groups and in the basolateral amygdala both mGluR1 and mGluR2 are expressed (Ohishi *et al.*, 1993; Shigemoto *et al.*, 1992). However, an action via Group I is more plausible since it is in agreement with the findings obtained both *in vivo* and *in vitro* showing Group I agonist-induced epileptic responses.

A popular model of epilepsy is electrical kindling, a phenomenon in which repeated application of low-intensity stimulation to amygdala or pyriform cortex causes gradual seizure development culminating in generalized motor seizures (Goddard *et al.*, 1969). Kindling, too, is blocked by injection of NMDA receptor antagonists (Croucher *et al.*, 1988; McNamara, 1988). There is accumulating evidence for a crucial role of presynaptic Groups II and III mGluR in this model (Attwell *et al.*, 1995; Suzuki *et al.*, 1996; Neugebauer *et al.*, 1997), but an involvement of postsynaptic Group I antagonists is not excluded (Suzuki *et al.*, 1996). Change in mGluR₁ and mGluR₅ mRNAs is seen in the hippocampus of amygdala-kindled rats (Akbar *et al.*, 1996), and alterations of both Groups I and II mGluR-mediated responses are found in amygdala neurons of kindled animals. Upregulation of Group I may contribute to the transition to epileptiform bursting in kindled cells (Holmes *et al.*, 1996). Activation of Group I mGluRs should be effective in modulating epileptic activity considering the exaggerated Ca^{2+} entry into kindled slices (Heinemann and Hamon, 1986). Blocking intracellular Ca^{2+} via Group I mGluR antagonism may interrupt the increased excitatory response of the epileptic neuron (Bradford, 1995), but this hypothesis awaits future studies and more specific mGluR agents.

5.3. Role of Group I mGluRs in Neurodegeneration

5.3.1. Brain Ischemia

Since glutamate plays a key role in ischemic brain damage, drugs that decrease the accumulation of glutamate or block its postsynaptic effects are associated with the amelioration of ischemic injury and should be regarded as potential therapeutic agents for stroke (Simon *et al.*, 1984; Rothman and Olney, 1986). NMDA receptors in particular have attracted the attention of researchers because they gate an ion channel permeable to Ca^{2+} and glutamate-induced neurotoxicity is mainly dependent on the intracellular accumulation of Ca^{2+} (Choi, 1988, 1995). Recent evidence has demonstrated that mGluRs, too, may play an important role in excitotoxicity (Nicoletti *et al.* (1996) for a review).

Quisqualate, DHPG or *L*-ADA amplify the excitotoxic neuronal degeneration induced by NMDA in cultured murine cortical cells (Bruno *et al.*, 1995a; Strasser *et al.*, 1998), suggesting that Group I mGluRs enhance NMDA receptor-mediated neuronal toxicity. The relief of Mg^{2+} blockade of NMDA channels by PKC has been proposed to be the principal process by which activation of Group I mGluRs amplifies NMDA toxicity (Bruno *et al.*, 1995a).

A neuroprotective effect against NMDA-mediated damage has been observed both by blocking Group I mGluRs (Buisson and Choi, 1995; Orlando *et al.*, 1995; Strasser *et al.*, 1998) and by activating Groups II and III mGluRs (Bruno *et al.*, 1994, 1995b; Buisson *et al.*, 1996). These results are in agreement with earlier experimental evidence of mixed effects using the non-selective mGluR agonist ACPD. This compound protects cultured neurons against excitotoxic degeneration and ischemic damage *in vivo* (Koh *et al.*, 1991; Chiamulera *et al.*, 1992; Siliprandi *et al.*, 1992; Pizzi *et al.*, 1993), but also produces neurotoxic effects when infused into the striatum or the hippocampus (Sacaan *et al.*, 1991; McDonald and Schoepp, 1992; Schoepp *et al.*, 1995). The neuroprotective action of ACPD is probably mediated by activation of presynaptic Groups II or III mGluRs (Bruno *et al.*, 1995b), whereas neuronal toxicity is mediated by Group I mGluRs.

Neurons in hippocampal CA1, a region known to be highly vulnerable to cerebral ischemia (Pulsinelli, 1985), are protected by MCPG from a hypoxic-hypoglycemic injury measured in slices electrophysiologically (Opitz *et al.*, 1994). A follow-up study using a variety of selective mGluR agents has shown that in this *in vitro* model of hypoxia, activation of Group I mGluR antagonism is beneficial if it happens before or during the insult (Opitz *et al.*, 1995). MCPG also attenuates the hypoxia-induced suppression of excitatory synaptic transmission in dentate gyrus neurons *in vitro* (Doherty and Dingledine, 1997).

A widely used model of global ischemia, a type of injury that typically occurs following cardiac arrest in humans, is the transient or permanent forebrain ischemia in gerbils produced by an occlusion of the carotid arteries (Araki *et al.*, 1989). The antagonist 4C3HPG protects CA1 pyramidal neurons when applied 20 min before permanent occlusion of the carotid arteries (Henrich-Noack *et al.*, 1998). As previously mentioned, 4C3HPG is also an agonist for Group II mGluRs, therefore the neuroprotective action could be due to activation of these receptors, or to a synergistic effect of both blocking Group I mGluRs and activating Group II mGluRs, as suggested by the authors (Henrich-Noack *et al.*, 1998). A role for Group II mGluRs in the gerbil model of global ischemia was partially confirmed with the specific mGluR₂ agonist (1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate (LY354740) (Bond *et al.*, 1998). In this study damage of CA1 cells induced by transient occlusion of the carotid arteries was reduced by LY354740. By increasing the severity of the ischemic insults, however, the neuroprotective effects were greatly reduced or disappeared.

Increased glutamate activation has been also implicated in another acute neurodegenerative disease, posttraumatic injury (Hayes *et al.*, 1988; Faden *et al.*, 1989). Pharmacological blockade of Group I mGluRs reduces neurological deficits produced by traumatic brain injury (Gong *et al.*, 1995), whereas activation of these receptors with DHPG exacerbates posttraumatic neuronal death in an *in vitro* model of cortical trauma (Mukhin *et al.*, 1996, 1997). In addition, an mGluR₁, but not mGluR₅,

antisense oligodeoxynucleotide is neuroprotective in this experimental model.

Oxidative stress represents an important pathway leading to neuronal degeneration which can interact with excessive glutamate activation to cause damage to brain tissue, including stroke, hypoxia, and trauma (Coyle and Puttfarcken, 1993). Recent data have shown that activation of Group I mGluRs in a hippocampal cell line generates a cellular response that is protective to oxidative stress caused by cysteine-deprivation or glucose starvation (Sagara and Schubert, 1998). In line with these results, mGluR₅ activation of cultured cerebellar granule cells also protects against apoptotic death (Copani *et al.*, 1995, 1998). The development of apoptosis was accelerated after treatment with either mGluR₅ antisense oligonucleotides or with the Group I mGluR antagonist MCPG. Interestingly, the functions of mGluR₅ in astrocytes support a role for these receptors in repair processes of injured CNS tissue (Balázs *et al.*, 1997). Programmed cell death or apoptosis can also occur in response to ischemia, or trauma, situations in which most cells die by necrosis as a result of acute injury. If injured cells do not die immediately, they may die by apoptosis (Raff *et al.*, 1993 for a review). Group I mGluRs, particularly mGluR₅, may contribute to the development of delayed death.

In summary, it appears that antagonism of Group I mGluRs, and particularly of mGluR₁, might be protective following brain ischemia and other forms of acute neuronal degenerative diseases like hypoxia/hypoglycemia (Opitz *et al.*, 1995) or traumatic brain injury (Mukhin *et al.*, 1996). Although lack of selectivity or brain penetration of the available compounds make it difficult to draw definite conclusions, it is already promising that blockade of mGluRs are effective in reducing neuronal damage after global ischemia, where NMDA receptor antagonists fail to do so (Buchan *et al.*, 1991; Rothman and Olney, 1995).

5.3.2. Huntington's and Parkinson's Diseases

Recently excitotoxicity has been proposed as participating in the pathogenesis of chronic neurodegenerative disorders such as Huntington's disease or Parkinson's disease (Albin and Greenamyre, 1992; Greenamyre and Porter, 1994; Blandini *et al.*, 1996). Consistent with this hypothesis, NMDA antagonists can attenuate the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced destruction of nigrostriatal neurons, a well known experimental model of Parkinson's disease (Turski *et al.*, 1991), and the NMDA receptor agonist, quinolinic acid, when injected into the striatum, causes lesions resembling the neuropathological features of Huntington's disease (Beal *et al.*, 1986).

The mGluR agonist ACPD injected in the striatum produces excitotoxicity (Sacaan *et al.*, 1991) and in the quinolinic acid model, the mixed Group I mGluR antagonist-Group II agonist 4C3HPG shows a protective action, similar to that produced by NMDA antagonists (Orlando *et al.*, 1995). In Parkinson's disease the degeneration of the dopaminergic neuronal system that projects from the

substantia nigra to the striatum is thought to lead to symptoms of the disease [Chase *et al.* (1998) for a review]. In the striatum, Sacaan *et al.* (1992) have demonstrated a functional interaction between DA and mGluRs. When ACPD was injected into the rat striatum, it induced extrapyramidal motor activation that was dependent on an intact DA system. Kearney *et al.* (1997) confirmed these results with the selective Group I agonist DHPG. Injections of ACPD or DHPG also activate the subthalamic nucleus, which provides excitatory input to the basal ganglia and influences locomotion and control of voluntary movements (Abbott *et al.*, 1997). Since overactivity of this nucleus is a major feature of Parkinson's disease (Albin *et al.*, 1989), Group I mGluR antagonists may provide a treatment for Parkinson's disease (Kearney *et al.*, 1997).

In the nucleus accumbens, an area in ventral striatum implicated in the limbic-motor interface [see Amalric *et al.* (1994) for a review], Group I mGluRs play an important role in the regulation of locomotor activity by interacting with dopaminergic neurotransmission (Attarian and Amalric, 1997; Kim and Vezina, 1997, 1998a). Recent findings have extended the contribution of Group I mGluRs in the nucleus accumbens to amphetamine-induced locomotion, suggesting a possible role for these receptors in the expression of sensitization behaviors induced by psychostimulant drugs (Kim and Vezina, 1998a,b).

Although research concerning the involvement of mGluRs in extrapyramidal motor disorders is still in its infancy, this beginning is surely very encouraging and in the next few years we can expect a great deal of progress in this area.

5.3.3. Alzheimer's Disease

The glutamatergic hypothesis of dementia was proposed in the late 1980s (Greenamyre *et al.*, 1988). According to this view, excitotoxicity participates in the pathogenesis of Alzheimer's disease (Greenamyre and Young, 1989; Choi, 1992). Interestingly, the β amyloid protein that accumulates in Alzheimer's disease can potentiate excitotoxic degeneration (Mattson *et al.*, 1992).

Stimulation of mGluR_{1a} in human embryonic kidney (HEK) 293 cells accelerates the breakdown of the amyloid precursor protein (APP) into non-amyloidogenic soluble forms of APPs, thus reducing β amyloid formation (Lee *et al.*, 1995). A similar effect is seen when cultured hippocampal neurons are stimulated with glutamate, quisqualate, or *trans*-ACPD. The effect is blocked by a PKC inhibitor and is mGluR-selective because iGluR agonists do not affect APPs degradation.

Agonists of Group I mGluRs might therefore represent a new class of therapeutic agents for Alzheimer's disease (Lee *et al.*, 1996). Furthermore, the involvement of Group I mGluRs in synaptic plasticity, such as LTP and learning and memory (see earlier), could lead to the development of specific agents that might modulate hippocampal functions to enhance cognitive functions in patients afflicted by dementia. Drugs acting on mGluRs might also show better results in clinical trials or

exhibit fewer side-effects than agents which block or modulate NMDA receptors [see Kornhuber and Wiltfang (1998) for a review].

5.4. Role of Group I mGluRs in Psychiatry Disorders

There are a number of clinical and animal laboratory reports suggesting an involvement of glutamate in psychiatric disorders. Indeed, glutamatergic abnormalities have been associated with schizophrenia (Bunney *et al.*, 1995; Olney and Farber, 1995) and mood disorders (Trullas and Skolnick, 1990; Skolnick *et al.*, 1996).

Recent evidence has demonstrated that treatment with the antidepressant imipramine can modify the sensitivity of Group I mGluRs, as measured by population spikes recorded in the CA1 region of the rat hippocampus (Pile *et al.*, 1998). Modification of Group I mGluRs may thus play a role in the mechanism of action of certain antidepressants.

The mGluRs can represent an alternative, non-dopaminergic therapy for the treatment of schizophrenia, as suggested by Moghaddam and Adams (1998) who found that a Group II specific mGluR agonist inhibiting glutamate release by modulating presynaptic sites, reversed the behavioral disruptions in the phenylclidine model of schizophrenia.

Future studies will show whether Group I postsynaptic mGluR antagonists also play a role in relieving schizophrenic symptoms. In general, mGluRs may provide important pharmacological therapeutic targets for psychiatric disorders in which glutamatergic neurotransmission is abnormally regulated. The metabotropic receptors have a clear advantage over the iGluRs which are ubiquitous and mediate fast synaptic transmission throughout the CNS (Moghaddam and Adams, 1998).

6. CONCLUSIONS

Our understanding of the functions of mGluRs in the brain has progressed in recent years at an exceptional pace. The study of mGluRs may be the fastest growing area of all neurotransmitter receptors. In particular, Group I mGluRs have been extensively investigated at the molecular, cellular, and behavioral level leading us to an appreciation of their great importance in the CNS.

Both mGluR₁ and mGluR₅ have been linked to a variety of brain disorders, including epilepsy, pain, ischemia and chronic neurodegenerative disorders. The role of Group I mGluRs in modulating glutamatergic neurotransmission and their selective presence in the CNS may provide a potential important therapeutic approach in many CNS disorders. iGluR treatment, on the other hand, had demonstrated in clinical trials to be associated with multiple side-effects possibly due to their ubiquity throughout the CNS and their fast excitatory properties.

Progress in developing drugs specific for the different mGluR classes requires, however, more selective and potent antagonists and agonists. With more compounds available, the next few years

should witness major advances in this area, adding to our understanding of mGluR functions in the brain and in the discovery of new agents for the treatment of neurological and psychiatric disorders.

Acknowledgements—The authors thank Professor E. Frank (Neurobiology, University of Pittsburgh, USA) and Dr M. Corsi for critical reading of earlier versions of the manuscript.

REFERENCES

- Aanonsen, L. M., Lei, S. and Wilcox, G. L. (1990) Excitatory amino acid receptors and nociceptive neurotransmission in rat spinal cord. *Pain* 41, 309–321.
- Abbott, A., Wigmore, M. A. and Lacey, M. G. (1997) Excitation of rat subthalamic nucleus neurones in vitro by activation of a group I metabotropic glutamate receptor. *Brain Res.* 766, 162–167.
- Abe, T., Sugihara, H., Nawa, H., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1992) Molecular characterization of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca²⁺ signal transduction. *J. Biol. Chem.* 267, 13361–13368.
- Aiba, A., Chen, C., Herrup, K., Rosenmund, C., Stevens, C. F. and Tonegawa, S. (1994a) Reduced hippocampal long-term potentiation and context-specific deficit in associative learning in mGluR1 mutant mice. *Cell* 79, 365–375.
- Aiba, A., Kano, M., Chen, C., Stanton, M. E., Fox, G. D., Herrup, K., Zwingman, T. A. and Tonegawa, S. (1994b) Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. *Cell* 79, 377–388.
- Akbar, M. T., Rattray, M., Powell, J. F. and Meldrum, B. S. (1996) Altered expression of group I metabotropic glutamate receptors in the hippocampus of amygdala-kindled rats. *Brain Res. Mol. Brain Res.* 43, 105–116.
- Albin, R. L. and Greenamyre, J. T. (1992) Alternative excitotoxic hypotheses. *Neurology* 42, 733–738.
- Albin, R. L., Young, A. B. and Penney, J. B. (1989) The functional anatomy of basal ganglia disorders. *Trends Neurosci.* 12, 366–375.
- Amalric, M., Ouagazzal, A., Baunez, C. and Nieoullon, A. (1994) Functional interactions between glutamate and dopamine in the rat striatum. *Neurochem. Int.* 25, 123–131.
- Aniksztejn, L., Otani, S. and Ben-Ari, Y. (1992) Quisqualate metabotropic receptors modulate NMDA currents and facilitate induction of long-term potentiation through protein kinase C. *Eur. J. Neurosci.* 4, 500–504.
- Annoura, H., Fukunaga, A., Uesugi, M., Tatsuoka, T. and Horikawa, Y. (1996) A novel class of antagonists for metabotropic glutamate receptors, 7-(hydroxyimino)-cyclopropa[b]chromen-1a-carboxylates. *Bioorg. Med. Chem. Lett.* 6, 763–766.
- Anwyl, R. (1991) The role of the metabotropic receptor in synaptic plasticity. *Trends Pharmac. Sci.* 12, 324–326.
- Araki, T., Kato, H. and Kogure, K. (1989) Selective neuronal vulnerability following transient cerebral ischemia in the gerbil: distribution and time course. *Acta Neurol. Scand.* 80, 548–553.
- Aramori, I. and Nakanishi, S. (1992) Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. *Neuron* 8, 757–765.
- Arvanov, V. L., Holmes, K. H., Keele, N. B. and Shinnick-Gallagher, P. (1995) The functional role of metabotropic glutamate receptors in epileptiform activity induced by 4-aminopyridine in the rat amygdala slice. *Brain Res.* 669, 140–144.
- Attarian, S. and Amalric, M. (1997) Microinfusion of the metabotropic glutamate receptor agonist 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid into the nucleus accumbens induces dopamine-dependent locomotor activation in the rat. *Eur. J. Neurosci.* 9, 809–816.
- Attwell, P. J., Kaura, S., Sigala, G., Bradford, H. F., Croucher, M. J., Jane, D. E. and Watkins, J. C. (1995) Blockade of both epileptogenesis and glutamate release by (1S,3S)-ACPD, a presynaptic glutamate receptor agonist. *Brain Res.* 698, 155–162.
- Balázs, R., Miller, S., Romano, C., de Vries, A., Chun, Y. and Cotman, C. W. (1997) Metabotropic glutamate receptor mGluR5 in astrocytes: pharmacological properties and agonist regulation. *J. Neurochem.* 69, 151–163.
- Balázs, R., Miller, S., Chun, Y., O'Toole, J. and Cotman, C. W. (1998) Metabotropic glutamate receptor agonists potentiate cyclic AMP formation induced by forskolin or beta-adrenergic receptor activation in cerebral cortical astrocytes in culture. *J. Neurochem.* 70, 2446–2458.
- Balschun, D. and Wetzel, W. (1998) Inhibition of group I metabotropic glutamate receptors blocks spatial learning in rats. *Neurosci. Lett.* 249, 41–44.
- Baranaukas, G. and Nistri, A. (1998) Sensitization of pain pathways in the spinal cord: cellular mechanisms. *Progr. Neurobiol.* 54, 349–365.
- Bashir, Z. I., Bortolotto, Z. A., Davies, C. H., Berretta, N., Irving, A. J., Seal, A. J., Henley, J. M., Jane, D. E., Watkins, J. C. and Collingridge, G. L. (1993) Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors. *Nature* 363, 347–350.
- Baskys, A. and Malenka, R. C. (1991) Agonists at metabotropic glutamate receptors presynaptically inhibit EPSCs in neonatal rat hippocampus. *J. Physiol. (Lond.)* 444, 687–701.
- Batchelor, A. M., Madge, D. J. and Garthwaite, J. (1994) Synaptic activation of metabotropic glutamate receptors in the parallel fibre-Purkinje cell pathway in rat cerebellar slices. *Neuroscience* 63, 911–915.
- Batchelor, A. M., Knopfel, T., Gasparini, F. and Garthwaite, J. (1997) Pharmacological characterization of synaptic transmission through mGluRs in rat cerebellar slices. *Neuropharmacology* 36, 401–403.
- Battaglia, G. and Rustioni, A. (1988) Coexistence of glutamate and substance P in dorsal root ganglion neurons of the rat and monkey. *J. Comp. Neurol.* 277, 302–312.
- Baude, A., Nusser, Z., Roberts, J. D., Mulvihill, E., McIlhinney, R. A. and Somogyi, P. (1993) The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. *Neuron* 11, 771–787.
- Beal, M. F., Kowall, N. W., Ellison, D. W., Mazurek, M. F., Swartz, K. J. and Martin, J. B. (1986) Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. *Nature* 321, 168–171.
- Bear, M. F. and Malenka, R. C. (1994) Synaptic plasticity: LTP and LTD. *Curr. Opin. Neurobiol.* 4, 389–399.
- Ben-Ari, Y. and Aniksztejn, L. (1995) Role of glutamate metabotropic receptors in long-term potentiation in the hippocampus. *Semin. Neurosci.* 7, 127–135.
- Besson, J. M. and Chaouch, A. (1987) Peripheral and spinal mechanisms of nociception. *Physiol. Rev.* 67, 67–186.
- Birse, E. F., Eaton, S. A., Jane, D. E., Jones, P. L., Porter, R. H., Pook, P. C., Sunter, D. C., Udvarhelyi, P. M., Wharton, B. and Roberts, P. J. et al. (1993) Phenylglycine derivatives as new pharmacological tools for investigating the role of metabotropic glutamate receptors in the central nervous system. *Neuroscience* 52, 481–488.
- Blandini, F., Porter, R. H. and Greenamyre, J. T. (1996) Glutamate and Parkinson's disease. *Mol. Neurobiol.* 12, 73–94.
- Bleakman, D., Rusin, K. I., Chard, P. S., Glaus, S. R. and Miller, R. J. (1992) Metabotropic glutamate receptors potentiate ionotropic glutamate responses in the rat dorsal horn. *Mol. Pharmacol.* 42, 192–196.
- Bliss, T. V. and Lomo, T. (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol. (Lond.)* 232, 331–356.
- Bliss, T. V. P. and Collingridge, G. L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361, 31–39.
- Blümcke, I., Behle, K., Malitschek, B., Kuhn, R., Knopfel, T., Wolf, H. K. and Wiestler, O. D. (1996) Immunohistochemical distribution of metabotropic glutamate receptor subtypes mGluR1b, mGluR2/3, mGluR4a and mGluR5 in human hippocampus. *Brain Res.* 736, 217–226.
- Bolshakov, V. Y. and Siegelbaum, S. A. (1994) Postsynaptic induction and presynaptic expression of hippocampal long-term depression. *Science* 264, 1148–1152.
- Bond, A. and Lodge, D. (1995) Pharmacology of metabotropic glutamate receptor-mediated enhancement of responses to excitatory and inhibitory amino acids on rat spinal neurones in vivo. *Neuropharmacology* 34, 1015–1023.
- Bond, A., O'Neill, M. J., Hicks, C. A., Monn, J. A. and Lodge, D. (1998) Neuroprotective effects of a systemically active group II metabotropic glutamate receptor agonist LY354740 in a gerbil model of global ischaemia. *Neuroreport* 9, 1191–1193.

- Bordi, F. (1996) Reduced long-term potentiation in the dentate gyrus of mGlu1 receptor-mutant mice in vivo. *Eur. J. Pharmac.* 301, R15-R16.
- Bordi, F. and Ugolini, A. (1995) Antagonists of the metabotropic glutamate receptor do not prevent induction of long-term potentiation in the dentate gyrus of rats. *Eur. J. Pharmac.* 273, 291-294.
- Bordi, F., Marcon, C., Chiamulera, C. and Reggiani, A. (1996) Effects of the metabotropic glutamate receptor antagonist MCPG on spatial and context-specific learning. *Neuropharmacology* 35, 1557-1565.
- Bordi, F., Reggiani, A. and Conquet, F. (1997) Regulation of synaptic plasticity by mGluR1 studied in vivo in mGluR1 mutant mice. *Brain Res.* 761, 121-126.
- Bortolotto, Z. A. and Collingridge, G. L. (1993) Characterisation of LTP induced by the activation of glutamate metabotropic receptors in area CA1 of the hippocampus. *Neuropharmacology* 32, 1-9.
- Bortolotto, Z. A. and Collingridge, G. L. (1995) On the mechanism of long-term potentiation induced by (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) in rat hippocampal slices. *Neuropharmacology* 34, 1003-1014.
- Bortolotto, Z. A., Bashir, Z. I., Davies, C. H. and Collingridge, G. L. (1994) A molecular switch activated by metabotropic glutamate receptors regulates induction of long-term potentiation. *Nature* 368, 740-743.
- Bortolotto, Z. A., Bashir, Z. I., Davies, C. H., Taira, T., Kaifa, K. and Collingridge, G. L. (1995) Studies on the role of metabotropic glutamate receptors in long-term potentiation: some methodological considerations. *J. Neurosci. Meth.* 59, 19-24.
- Boxall, S. J., Thompson, S. W., Dray, A., Dickenson, A. H. and Urban, L. (1996) Metabotropic glutamate receptor activation contributes to nociceptive reflex activity in the rat spinal cord in vitro. *Neuroscience* 74, 13-20.
- Brabet, I., Mary, S., Bockaert, J. and Pin, J. P. (1995) Phenylglycine derivatives discriminate between mGluR1- and mGluR5-mediated responses. *Neuropharmacology* 34, 895-903.
- Bradford, H. F. (1995) Glutamate, GABA and epilepsy. *Progr. Neurobiol.* 47, 477-511.
- Breakwell, N. A., Rowan, M. J. and Anwyl, R. (1996) Metabotropic glutamate receptor dependent EPSP and EPSP-spike potentiation in area CA1 of the submerged rat hippocampal slice. *J. Neurophysiol.* 76, 3126-3135.
- Bruno, V., Copani, A., Battaglia, G., Raffaele, R., Shinozaki, H. and Nicoletti, F. (1994) Protective effect of the metabotropic glutamate receptor agonist, DCG-IV, against excitotoxic neuronal death. *Eur. J. Pharmac.* 256, 109-112.
- Bruno, V., Battaglia, G., Copani, A., Giffard, R. G., Raciti, G., Raffaele, R., Shinozaki, H. and Nicoletti, F. (1995a) Activation of class II or III metabotropic glutamate receptors protects cultured cortical neurons against excitotoxic degeneration. *Eur. J. Neurosci.* 7, 1906-1913.
- Bruno, V., Copani, A., Knopfel, T., Kuhn, R., Casabona, G., Dell'Albani, P., Condorelli, D. F. and Nicoletti, F. (1995b) Activation of metabotropic glutamate receptors coupled to inositol phospholipid hydrolysis amplifies NMDA-induced neuronal degeneration in cultured cortical cells. *Neuropharmacology* 34, 1089-1098.
- Buchan, A., Li, H. and Pulsinelli, W. A. (1991) The N-methyl-D-aspartate antagonist, MK-801, fails to protect against neuronal damage caused by transient, severe forebrain ischemia in adult rats. *J. Neurosci.* 11, 1049-1056.
- Buckmaster, P. S. and Schwartzkroin, P. A. (1995) Interneurons and inhibition in the dentate gyrus of the rat in vivo. *J. Neurosci.* 15, 774-789.
- Budai, D. and Larson, A. A. (1998) The involvement of metabotropic glutamate receptors in sensory transmission in dorsal horn of the rat spinal cord. *Neuroscience* 83, 571-580.
- Budd, D. C. and Nicholls, D. G. (1998) Arachidonic acid potentiates the duration of the metabotropic, protein kinase C-mediated, suppression of the inhibitory adenosine A1 receptor pathway in glutamatergic nerve terminals from rat cerebral cortex [In Process Citation]. *Neurosci. Lett.* 244, 133-136.
- Buisson, A. and Choi, D. W. (1995) The inhibitory mGluR agonist, S-4-carboxy-3-hydroxy-phenylglycine selectively attenuates NMDA neurotoxicity and oxygen-glucose deprivation-induced neuronal death. *Neuropharmacology* 34, 1081-1087.
- Buisson, A., Yu, S. P. and Choi, D. W. (1996) DCG-IV selectively attenuates rapidly triggered NMDA-induced neurotoxicity in cortical neurons. *Eur. J. Neurosci.* 8, 138-143.
- Bunney, B. G., Bunney, W. E. and Carlsson, A. (1995) Schizophrenia and Glutamate. In: *Psychopharmacology: The Fourth Generation of Progress*, pp. 1205-1214. Eds. F. E. Bloom and D. J. Kupfer. Raven Press, New York.
- Burke, J. P. and Hablitz, J. J. (1994) Presynaptic depression of synaptic transmission mediated by activation of metabotropic glutamate receptors in rat neocortex. *J. Neurosci.* 14, 5120-5130.
- Burke, J. P. and Hablitz, J. J. (1995) Modulation of epileptiform activity by metabotropic glutamate receptors in immature rat neocortex. *J. Neurophysiol.* 73, 205-217.
- Burke, J. P. and Hablitz, J. J. (1996) G-protein activation by metabotropic glutamate receptors reduces spike frequency adaptation in neocortical neurons. *Neuroscience* 75, 123-131.
- Cahusac, P. M. (1994) Cortical layer-specific effects of the metabotropic glutamate receptor agonist 1S,3R-ACPD in rat primary somatosensory cortex in vivo. *Eur. J. Neurosci.* 6, 1505-1511.
- Calabresi, P., Mercuri, N. B. and Bernardi, G. (1992) Activation of quisqualate metabotropic receptors reduces glutamate and GABA-mediated synaptic potentials in the rat striatum. *Neurosci. Lett.* 139, 41-44.
- Camón, L., Vives, P., de Vera, N. and Martinez, E. (1998) Seizures and neuronal damage induced in the rat by activation of group I metabotropic glutamate receptors with their selective agonist 3,5-dihydroxyphenylglycine. *J. Neurosci. Res.* 51, 339-348.
- Carmant, L., Woodhall, G., Ouardouz, M., Robitaille, R. and Lacaille, J. C. (1997) Interneuron-specific Ca²⁺ responses linked to metabotropic and ionotropic glutamate receptors in rat hippocampal slices. *Eur. J. Neurosci.* 9, 1625-1635.
- Cerne, R. and Randic, M. (1992) Modulation of AMPA and NMDA responses in rat spinal dorsal horn neurons by trans-1-aminocyclopentane-1,3-dicarboxylic acid. *Neurosci. Lett.* 144, 180-184.
- Chapman, A. G., Smith, S. E. and Meldrum, B. S. (1991) The anticonvulsant effect of the non-NMDA antagonists, NBQX and GYKI 52466, in mice. *Epilepsy Res.* 9, 92-96.
- Charpak, S., Gahwiler, B. H., Do, K. Q. and Knopfel, T. (1990) Potassium conductances in hippocampal neurons blocked by excitatory amino-acid transmitters. *Nature* 347, 765-767.
- Chase, T. N., Oh, J. D. and Blanchet, P. J. (1998) Neostriatal mechanisms in Parkinson's disease. *Neurology* 51, S30-S35.
- Chavis, P., Fagni, L., Bockaert, J. and Lansman, J. B. (1995) Modulation of calcium channels by metabotropic glutamate receptors in cerebellar granule cells. *Neuropharmacology* 34, 929-937.
- Chavis, P., Fagni, L., Lansman, J. B. and Bockaert, J. (1996) Functional coupling between ryanodine receptors and L-type calcium channels in neurons. *Nature* 382, 719-722.
- Chiamulera, C., Albertini, P., Valerio, E. and Reggiani, A. (1992) Activation of metabotropic receptors has a neuroprotective effect in a rodent model of focal ischaemia. *Eur. J. Pharmac.* 216, 335-336.
- Chinestra, P., Aniksztejn, L., Diabira, D. and Ben-Ari, Y. (1993) (RS)-alpha-methyl-4-carboxyphenylglycine neither prevents induction of LTP nor antagonizes metabotropic glutamate receptors in CA1 hippocampal neurons. *J. Neurophysiol.* 70, 2684-2689.
- Choi, D. W. (1988) Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci.* 11, 465-469.
- Choi, D. W. (1992) Bench to bedside: the glutamate connection. *Science* 258, 241-243.
- Choi, D. W. (1995) Calcium: still center-stage in hypoxic-ischemic neuronal death. *Trends Neurosci.* 18, 58-60.
- Choi, S. and Lovinger, D. M. (1996) Metabotropic glutamate receptor modulation of voltage-gated Ca²⁺ channels involves multiple receptor subtypes in cortical neurons. *J. Neurosci.* 16, 36-45.
- Chu, Z. and Hablitz, J. J. (1998) Activation of group I mGluRs increases spontaneous IPSC frequency in rat frontal cortex. *J. Neurophysiol.* 80, 621-627.
- Clark, B. P., Baker, S. R., Goldworthy, J., Harris, J. R. and Kingston, A. E. (1997) (+)-2-Methyl-4-carboxyphenylglycine (LY367385) selectively antagonises glutamate mGluR1 receptors. *Bioorg. Med. Chem. Lett.* 7, 2777-2780.
- Coderre, T. J. and Melzack, R. (1992) The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. *J. Neurosci.* 12, 3665-3670.
- Coderre, T. J., Vaccarino, A. L. and Melzack, R. (1990) Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. *Brain Res.* 535, 155-158.

- Colwell, C. S. and Levine, M. S. (1994) Metabotropic glutamate receptors modulate N-methyl-D-aspartate receptor function in neostriatal neurons. *Neuroscience* 61, 497-507.
- Congar, P., Leinekugel, X., Ben-Ari, Y. and Crépel, F. (1997) A long-lasting calcium-activated nonselective cationic current is generated by synaptic stimulation or exogenous activation of group I metabotropic glutamate receptors in CA1 pyramidal neurons. *J. Neurosci.* 17, 5366-5379.
- Conn, P. J. and Pin, J. P. (1997) Pharmacology and functions of metabotropic glutamate receptors. *A. Rev. Pharmac. Toxic.* 37, 205-237.
- Conquet, F., Bashir, Z. I., Davies, C. H., Daniel, H., Ferraguti, F., Bordi, F., Franz-Bacon, K., Reggiani, A., Matarese, V., Condé, F., Collingridge, G. L., Crépel, F. (1994) Motor deficit and impairment of synaptic plasticity in mice lacking mGluR1. *Nature* 372, 237-243.
- Copani, A., Bruno, V. M., Barresi, V., Battaglia, G., Condorelli, D. F. and Nicoletti, F. (1995) Activation of metabotropic glutamate receptors prevents neuronal apoptosis in culture. *J. Neurochem.* 64, 101-108.
- Copani, A., Casabona, G., Bruno, V., Caruso, A., Condorelli, D. F., Messina, A., Di Giorgi Gerevini, V., Pin, J. P., Kuhn, R., Knopfel, T., Nicoletti, F. (1998) The metabotropic glutamate receptor mGlu5 controls the onset of developmental apoptosis in cultured cerebellar neurons [In Process Citation]. *Eur. J. Neurosci.* 10, 2173-2184.
- Corsi, M., Ugolini, A., Quartaroli, M., Chiamulera, C., Corti, C., Maraia, G., Conquet, F. and Ferraguti, F. (1997) Phospholipase C-coupled metabotropic glutamate receptors modulate nociceptive transmission. In: *Metabotropic Glutamate Receptors and Brain Functions*, pp. 37-48. Eds. F. Moroni, F. Nicoletti and D. E. Pellegrini-Giampietro. Portland Press, London.
- Coyle, J. T. and Puttfarcken, P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262, 689-695.
- Crépel, V., Aniksztejn, L., Ben-Ari, Y. and Hammond, C. (1994) Glutamate metabotropic receptors increase a Ca^{2+} -activated nonspecific cationic current in CA1 hippocampal neurons. *J. Neurophysiol.* 72, 1561-1569.
- Croucher, M. J., Bradford, H. F., Sunter, D. C. and Watkins, J. C. (1988) Inhibition of the development of electrical kindling of the prepyriform cortex by daily focal injections of excitatory amino acid antagonists. *Eur. J. Pharmac.* 152, 29-38.
- Dalby, N. O. and Thomsen, C. (1996) Modulation of seizure activity in mice by metabotropic glutamate receptor ligands. *J. Pharmac. exp. Ther.* 276, 516-522.
- Daniel, H., Hemart, N., Jaillard, D. and Crépel, F. (1992) Coactivation of metabotropic glutamate receptors and of voltage-gated calcium channels induces long-term depression in cerebellar Purkinje cells in vitro. *Exp. Brain Res.* 90, 327-331.
- Davies, C. H., Clarke, V. R., Jane, D. E. and Collingridge, G. L. (1995) Pharmacology of postsynaptic metabotropic glutamate receptors in rat hippocampal CA1 pyramidal neurones. *Br. J. Pharmac.* 116, 1859-1869.
- Davies, S. N. and Lodge, D. (1987) Evidence for involvement of N-methylaspartate receptors in 'wind-up' of class 2 neurones in the dorsal horn of the rat. *Brain Res.* 424, 402-406.
- Desai, M. A. and Conn, P. J. (1991) Excitatory effects of ACPD receptor activation in the hippocampus are mediated by direct effects on pyramidal cells and blockade of synaptic inhibition. *J. Neurophysiol.* 66, 40-52.
- Desai, M. A., Smith, T. S. and Conn, P. J. (1992) Multiple metabotropic glutamate receptors regulate hippocampal function. *Synapse* 12, 206-213.
- Desai, M. A., McBain, C. J., Kauer, J. A. and Conn, P. J. (1994) Metabotropic glutamate receptor-induced disinhibition is mediated by reduced transmission at excitatory synapses onto interneurons and inhibitory synapses onto pyramidal cells. *Neurosci. Lett.* 181, 78-82.
- Dickenson, A. H. (1995) Central acute pain mechanisms. *Ann. Med.* 27, 223-227.
- Dickenson, A. H. and Sullivan, A. F. (1987) Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation. *Neuropharmacology* 26, 1235-1238.
- Dickenson, A. H., Chapman, V. and Green, G. M. (1997) The pharmacology of excitatory and inhibitory amino acid-mediated events in the transmission and modulation of pain in the spinal cord. *Gen. Pharmac.* 28, 633-638.
- Doherty, J. and Dingledine, R. (1997) Regulation of excitatory input to inhibitory interneurons of the dentate gyrus during hypoxia. *J. Neurophysiol.* 77, 393-404.
- Doherty, J. and Dingledine, R. (1998) Differential regulation of synaptic inputs to dentate hilar border interneurons by metabotropic glutamate receptors. *J. Neurophysiol.* 79, 2903-2910.
- Doherty, A. J., Palmer, M. J., Henley, J. M., Collingridge, G. L. and Jane, D. E. (1997) (RS)-2-Chloro-5-hydroxyphenylglycine (CHPG) activates mGlu5, but no mGlu1, receptors expressed in CHO cells and potentiates NMDA responses in the hippocampus. *Neuropharmacology* 36, 265-267.
- Dorri, F., Hampson, D. R., Baskys, A. and Wojtowicz, J. M. (1997) Down-regulation of mGluR5 by antisense deoxynucleotides alters pharmacological responses to applications of ACPD in the rat hippocampus. *Exp. Neurol.* 147, 48-54.
- Eaton, S. A., Birse, E. F., Wharton, B., Sunter, D. C., Udvarhelyi, P. M., Watkins, J. C. and Salt, T. E. (1993a) Mediation of thalamic sensory responses in vivo by ACPD-activated excitatory amino acid receptors. *Eur. J. Neurosci.* 5, 186-189.
- Eaton, S. A., Jane, D. E., Jones, P. L., Porter, R. H., Pook, P. C., Sunter, D. C., Udvarhelyi, P. M., Roberts, P. J., Salt, T. E. and Watkins, J. C. (1993b) Competitive antagonism at metabotropic glutamate receptors by (S)-4-carboxyphenylglycine and (RS)-alpha-methyl-4-carboxyphenylglycine. *Eur. J. Pharmac.* 244, 195-197.
- Faden, A. I., Demediuk, P., Panter, S. S. and Vink, R. (1989) The role of excitatory amino acids and NMDA receptors in traumatic brain injury. *Science* 244, 798-800.
- Fisher, K. andCoderre, T. J. (1996a) Comparison of nociceptive effects produced by intrathecal administration of mGluR agonists. *Neuroreport* 7, 2743-2747.
- Fisher, K. andCoderre, T. J. (1996b) The contribution of metabotropic glutamate receptors (mGluRs) to formalin-induced nociception. *Pain* 68, 255-263.
- Fisher, K., Fundytus, M. E., Cahill, C. M. andCoderre, T. J. (1998) Intrathecal administration of the mGluR compound, (S)-4CPG, attenuates hyperalgesia and allodynia associated with sciatic nerve constriction injury in rats. *Pain* 77, 59-66.
- Fitzjohn, S. M., Irving, A. J., Palmer, M. J., Harvey, J., Lodge, D. and Collingridge, G. L. (1996) Activation of group I mGluRs potentiates NMDA responses in rat hippocampal slices [published erratum appears in *Neurosci. Lett.* 1996 Mar 29;207(2):142]. *Neurosci. Lett.* 203, 211-213.
- Fotuhi, M., Sharp, A. H., Glatt, C. E., Hwang, P. M., von Krosigk, M., Snyder, S. H. and Dawson, T. M. (1993) Differential localization of phosphoinositide-linked metabotropic glutamate receptor (mGluR1) and the inositol 1,4,5-trisphosphate receptor in rat brain. *J. Neurosci.* 13, 2001-2012.
- Fundytus, M. E. andCoderre, T. J. (1994) Effect of activity at metabotropic, as well as ionotropic (NMDA), glutamate receptors on morphine dependence. *Br. J. Pharmac.* 113, 1215-1220.
- Fundytus, M. E. andCoderre, T. J. (1997) Attenuation of precipitated morphine withdrawal symptoms by acute i.c.v. administration of a group II mGluR agonist. *Br. J. Pharmac.* 121, 511-514.
- Fundytus, M. E., Ritchie, J. andCoderre, T. J. (1997) Attenuation of morphine withdrawal symptoms by subtype-selective metabotropic glutamate receptor antagonists. *Br. J. Pharmac.* 120, 1015-1020.
- Fundytus, M. E., Fisher, K., Dray, A., Henry, J. L. andCoderre, T. J. (1998) In vivo antinociceptive activity of anti-rat mGluR1 and mGluR5 antibodies in rats. *Neuroreport* 9, 731-735.
- Gerber, U. andGähwiler, B. H. (1994) Modulation of Ionic Currents by Metabotropic Glutamate Receptors in the CNS. In: *The Metabotropic Glutamate Receptors*, pp. 125-146. Eds. P. J. Conn and J. Patel. Humana Press, Totowa, NJ.
- Gereau, R. W. t. and Conn, P. J. (1995a) Multiple presynaptic metabotropic glutamate receptors modulate excitatory and inhibitory synaptic transmission in hippocampal area CA1. *J. Neurosci.* 15, 6879-6889.
- Gereau, R. W. t. and Conn, P. J. (1995b) Roles of specific metabotropic glutamate receptor subtypes in regulation of hippocampal CA1 pyramidal cell excitability. *J. Neurophysiol.* 74, 122-129.
- Glaum, S. R. and Miller, R. J. (1992) Metabotropic glutamate receptors mediate excitatory transmission in the nucleus of the solitary tract. *J. Neurosci.* 12, 2251-2258.
- Glaum, S. R. and Miller, R. J. (1993) Activation of metabotropic glutamate receptors produces reciprocal regulation of ionotropic glutamate and GABA responses in the nucleus of the tractus solitarius of the rat. *J. Neurosci.* 13, 1636-1641.
- Glaum, S. R. and Miller, R. J. (1994) Acute regulation of synaptic transmission by metabotropic glutamate receptors. In: *The Metabotropic Glutamate Receptors*, pp. 147-172. Eds. P. J. Conn and J. Patel. Humana Press, Totowa, NJ.

- Goddard, G. V., McIntyre, D. C. and Leech, C. K. (1969) A permanent change in brain function resulting from daily electrical stimulation. *Exp. Neurol.* **25**, 295-330.
- Gong, Q. Z., Delahunty, T. M., Hamm, R. J. and Lyeth, B. G. (1995) Metabotropic glutamate antagonist, MCPG, treatment of traumatic brain injury in rats. *Brain Res.* **700**, 299-302.
- Greenamyre, J. T. and Porter, R. H. (1994) Anatomy and physiology of glutamate in the CNS. *Neurology* **44**, S7-S13.
- Greenamyre, J. T. and Young, A. B. (1989) Excitatory amino acids and Alzheimer's disease. *Neurobiol. Aging* **10**, 593-602.
- Greenamyre, J. T., Maragos, W. F., Albin, R. L., Penney, J. B. and Young, A. B. (1988) Glutamate transmission and toxicity in Alzheimer's disease. *Progr. Neuropsychopharmac. Biol. Psychiat.* **12**, 421-430.
- Guérineau, N. C., Bossu, J. L., Gähwiler, B. H. and Gerber, U. (1995) Activation of a nonselective cationic conductance by metabotropic glutamatergic and muscarinic agonists in CA3 pyramidal neurons of the rat hippocampus. *J. Neurosci.* **15**, 4395-4407.
- Hampson, D. R., Theriault, E., Huang, X. P., Kristensen, P., Pickering, D. S., Franck, J. E. and Mulvihill, E. R. (1994) Characterization of two alternatively spliced forms of a metabotropic glutamate receptor in the central nervous system of the rat. *Neuroscience* **60**, 325-336.
- Hartell, N. A. (1994) Induction of cerebellar long-term depression requires activation of glutamate metabotropic receptors. *Neuroreport* **5**, 913-916.
- Haruta, H., Kamishita, T., Hicks, T. P., Takahashi, M. P. and Tsumoto, T. (1994) Induction of LTD but not LTP through metabotropic glutamate receptors in visual cortex. *Neuroreport* **5**, 1829-1832.
- Harvey, J. and Collingridge, G. L. (1993) Signal transduction pathways involved in the acute potentiation of NMDA responses by 1S,3R-ACPD in rat hippocampal slices. *Br. J. Pharmac.* **109**, 1085-1090.
- Harvey, J., Palmer, M. J., Irving, A. J., Clarke, V. R. and Collingridge, G. L. (1996) NMDA receptor dependence of mGlu-mediated depression of synaptic transmission in the CA1 region of the rat hippocampus. *Br. J. Pharmac.* **119**, 1239-1247.
- Hayashi, Y., Momiyama, A., Takahashi, T., Ohishi, H., Ogawa-Meguro, R., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1993) Role of a metabotropic glutamate receptor in synaptic modulation in the accessory olfactory bulb. *Nature* **366**, 687-690.
- Hayashi, Y., Sekiyama, N., Nakanishi, S., Jane, D. E., Sunter, D. C., Birse, E. F., Udvarhelyi, P. M. and Watkins, J. C. (1994) Analysis of agonist and antagonist activities of phenylglycine derivatives for different cloned metabotropic glutamate receptor subtypes. *J. Neurosci.* **14**, 3370-3377.
- Hayes, R. L., Jenkins, L. W., Lyeth, B. G., Balster, R. L., Robinson, S. E., Clifton, G. L., Stubbs, J. F. and Young, H. F. (1988) Pretreatment with phencyclidine, an N-methyl-D-aspartate antagonist, attenuates long-term behavioral deficits in the rat produced by traumatic brain injury. *J. Neurotrauma* **5**, 259-274.
- Heinemann, U. and Hamon, B. (1986) Calcium and epileptogenesis. *Exp. Brain Res.* **65**, 1-10.
- Henrich-Noack, P., Hatton, C. D. and Reymann, K. G. (1998) The mGlu receptor ligand (S)-4C3HPG protects neurons after global ischaemia in gerbils. *Neuroreport* **9**, 985-988.
- Hensch, T. K. and Stryker, M. P. (1996) Ocular dominance plasticity under metabotropic glutamate receptor blockade. *Science* **272**, 554-557.
- Hollmann, M. and Heinemann, S. (1994) Cloned glutamate receptors. *A. Rev. Neurosci.* **17**, 31-108.
- Holmes, K. H., Keele, N. B. and Shinnick-Gallagher, P. (1996) Loss of mGluR-mediated hyperpolarizations and increase in mGluR depolarizations in basolateral amygdala neurons in kindling-induced epilepsy. *J. Neurophysiol.* **76**, 2808-2812.
- Holscher, C. (1994) Inhibitors of metabotropic glutamate receptors produce amnesic effects in chicks. *Neuroreport* **5**, 1037-1040.
- Houamed, K. M., Kuijper, J. L., Gilbert, T. L., Haldeman, B. A., O'Hara, P. J., Mulvihill, E. R., Almers, W. and Hagen, F. S. (1991) Cloning, expression, and gene structure of a G protein-coupled glutamate receptor from rat brain. *Science* **252**, 1318-1321.
- Hsia, A. Y., Salin, P. A., Castillo, P. E., Aiba, A., Abeliovich, A., Tonegawa, S. and Nicoll, R. A. (1995) Evidence against a role for metabotropic glutamate receptors in mossy fiber LTP: the use of mutant mice and pharmacological antagonists. *Neuropharmacology* **34**, 1567-1572.
- Huber, K. M., Sawtell, N. B. and Bear, M. F. (1998) Effects of the metabotropic glutamate receptor antagonist MCPG on phosphoinositide turnover and synaptic plasticity in visual cortex. *J. Neurosci.* **18**, 1-9.
- Irving, A. J., Schofield, J. G., Watkins, J. C., Sunter, D. C. and Collingridge, G. L. (1990) 1S,3R-ACPD stimulates and L-AP3 blocks Ca²⁺ mobilization in rat cerebellar neurons. *Eur. J. Pharmac.* **186**, 363-365.
- Ito, I., Kohda, A., Tanabe, S., Hirose, E., Hayashi, M., Mitsunaga, S. and Sugiyama, H. (1992) 3,5-Dihydroxyphenylglycine: a potent agonist of metabotropic glutamate receptors. *Neuroreport* **3**, 1013-1016.
- Izumi, Y. and Zorumski, C. F. (1994) Developmental changes in the effects of metabotropic glutamate receptor antagonists on CA1 long-term potentiation in rat hippocampal slices. *Neurosci. Lett.* **176**, 89-92.
- Jane, D. E., Jones, P. L., Pook, P. C., Salt, T. E., Sunter, D. C. and Watkins, J. C. (1993) Stereospecific antagonism by (+)-alpha-methyl-4-carboxyphenylglycine (MCPG) of (1S,3R)-ACPD-induced effects in neonatal rat motoneurons and rat thalamic neurons. *Neuropharmacology* **32**, 725-727.
- Jane, D. E., Jones, P. L., Pook, P. C., Tse, H. W. and Watkins, J. C. (1994) Actions of two new antagonists showing selectivity for different sub-types of metabotropic glutamate receptor in the neonatal rat spinal cord. *Br. J. Pharmac.* **112**, 809-816.
- Joly, C., Gomez, J., Brabet, I., Curry, K., Bockaert, J. and Pin, J. P. (1995) Molecular, functional, and pharmacological characterization of the metabotropic glutamate receptor type 5 splice variants: comparison with mGluR1. *J. Neurosci.* **15**, 3970-3981.
- Jones, M. W. and Headley, P. M. (1995) Interactions between metabotropic and ionotropic glutamate receptor agonists in the rat spinal cord in vivo. *Neuropharmacology* **34**, 1025-1031.
- Jones, P. L. S. J., Birse, E. F., Jane, D. E., Jones, A. W., Mewett, K. N., Pook, P. C.-K., Udvarhelyi, P. M., Wharton, B. and Watkins, J. C. (1993) Agonist and antagonist actions of the phenylglycine derivatives at depolarization-mediated (1S,3R)-ACPD receptors in neonatal rat motoneurons. *Br. J. Pharmac.* **108**, 86P.
- Jouvenceau, A., Dutar, P. and Billard, J. M. (1995) Presynaptic depression of inhibitory postsynaptic potentials by metabotropic glutamate receptors in rat hippocampal CA1 pyramidal cells. *Eur. J. Pharmac.* **281**, 131-139.
- Kaba, H., Hayashi, Y., Higuchi, T. and Nakanishi, S. (1994) Induction of an olfactory memory by the activation of a metabotropic glutamate receptor. *Science* **265**, 262-264.
- Kato, N. (1993) Dependence of long-term depression on postsynaptic metabotropic glutamate receptors in visual cortex. *Proc. natl Acad. Sci. USA* **90**, 3650-3654.
- Kawabata, S., Tsutsumi, R., Kohara, A., Yamaguchi, T., Nakanishi, S. and Okada, M. (1996) Control of calcium oscillations by phosphorylation of metabotropic glutamate receptors. *Nature* **383**, 89-92.
- Kearney, J. A., Frey, K. A. and Albin, R. L. (1997) Metabotropic glutamate agonist-induced rotation: a pharmacological, FOS immunohistochemical, and [14C]-2-deoxyglucose autoradiographic study. *J. Neurosci.* **17**, 4415-4425.
- Keele, N. B., Arvanov, V. L. and Shinnick-Gallagher, P. (1997) Quisqualate-preferring metabotropic glutamate receptor activates Na(+)-Ca²⁺ exchange in rat basolateral amygdala neurons. *J. Physiol. (Lond.)* **499**, 87-104.
- Kelso, S. R., Nelson, T. E. and Leonard, J. P. (1992) Protein kinase C-mediated enhancement of NMDA currents by metabotropic glutamate receptors in *Xenopus* oocytes. *J. Physiol. (Lond.)* **449**, 705-718.
- Kim, J. H. and Vezina, P. (1997) Activation of metabotropic glutamate receptors in the rat nucleus accumbens increases locomotor activity in a dopamine-dependent manner. *J. Pharmac. exp. Ther.* **283**, 962-968.
- Kim, J. H. and Vezina, P. (1998a) The metabotropic glutamate receptor antagonist (RS)-MCPG produces hyperlocomotion in amphetamine pre-exposed rats. *Neuropharmacology* **37**, 189-197.
- Kim, J. H. and Vezina, P. (1998b) Metabotropic glutamate receptors are necessary for sensitization by amphetamine. *Neuroreport* **9**, 403-406.
- King, A. E., Thompson, S. W., Urban, L. and Woolf, C. J. (1988) The responses recorded in vitro of deep dorsal horn neurons to direct and orthodromic stimulation in the young rat spinal cord. *Neuroscience* **27**, 231-242.
- Kinney, G. A. and Slater, N. T. (1993) Potentiation of NMDA receptor-mediated transmission in turtle cerebellar granule cells by activation of metabotropic glutamate receptors. *J. Neurophysiol.* **69**, 585-594.

- Koh, J. Y., Palmer, E. and Cotman, C. W. (1991) Activation of the metabotropic glutamate receptor attenuates N-methyl-D-aspartate neurotoxicity in cortical cultures. *Proc. natl Acad. Sci. U.S.A.* **88**, 9431-9435.
- Kornhuber, J. and Wiltfang, J. (1998) The role of glutamate in dementia. *J. Neural Transm. Suppl.* **53**, 277-287.
- Lee, R. K., Wurtman, R. J., Cox, A. J. and Nitsch, R. M. (1995) Amyloid precursor protein processing is stimulated by metabotropic glutamate receptors. *Proc. natl Acad. Sci. U.S.A.* **92**, 8083-8087.
- Lee, R. K., Jimenez, J., Cox, A. J. and Wurtman, R. J. (1996) Metabotropic glutamate receptors regulate APP processing in hippocampal neurons and cortical astrocytes derived from fetal rats. *Ann. N.Y. Acad. Sci.* **777**, 338-343.
- Lester, R. A. and Jahr, C. E. (1990) Quisqualate receptor-mediated depression of calcium currents in hippocampal neurons. *Neuron* **4**, 741-749.
- Libri, V., Constanti, A., Zibetti, M. and Postlethwaite, M. (1997) Metabotropic glutamate receptor subtypes mediating slow inward tail current (IADP) induction and inhibition of synaptic transmission in olfactory cortical neurones. *Br. J. Pharmac.* **120**, 1083-1095.
- Linden, D. J. and Connor, J. A. (1993) Cellular mechanisms of long-term depression in the cerebellum. *Curr. Opin. Neurobiol.* **3**, 401-406.
- Lingenhöhl, K., Olpe, H. R., Bendali, N. and Knöpfel, T. (1993) Phenylglycine derivatives antagonize the excitatory response to Purkinje cells to 1S,3R-ACPD: an in vivo and in vitro study. *Neurosci. Res.* **18**, 229-234.
- Little, Z., Grover, L. M. and Teyler, T. J. (1995) Metabotropic glutamate receptor antagonist, (R,S)-alpha-methyl-4-carboxyphenylglycine, blocks two distinct forms of long-term potentiation in area CA1 of rat hippocampus. *Neurosci. Lett.* **201**, 73-76.
- Liu, Y. B., Disterhoft, J. F. and Slater, N. T. (1993) Activation of metabotropic glutamate receptors induces long-term depression of GABAergic inhibition in hippocampus. *J. Neurophysiol.* **69**, 1000-1004.
- Lu, Y. M., Jia, Z., Janus, C., Henderson, J. T., Gerlai, R., Wojtowicz, J. M. and Roder, J. C. (1997) Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. *J. Neurosci.* **17**, 5196-5205.
- Luján, R., Nusser, Z., Roberts, J. D., Shigemoto, R. and Somogyi, P. (1996) Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. *Eur. J. Neurosci.* **8**, 1488-1500.
- Lüthi, A., Gähwiler, B. H. and Gerber, U. (1997) 1S, 3R-ACPD induces a region of negative slope conductance in the steady-state current-voltage relationship of hippocampal pyramidal cells. *J. Neurophysiol.* **77**, 221-228.
- Malenka, R. C., Kauer, J. A., Zucker, R. S. and Nicoll, R. A. (1988) Postsynaptic calcium is sufficient for potentiation of hippocampal synaptic transmission. *Science* **242**, 81-84.
- Malenka, R. C., Lancaster, B. and Zucker, R. S. (1992) Temporal limits on the rise in postsynaptic calcium required for the induction of long-term potentiation. *Neuron* **9**, 121-128.
- Manahan-Vaughan, D. (1997) Group I and 2 metabotropic glutamate receptors play differential roles in hippocampal long-term depression and long-term potentiation in freely moving rats. *J. Neurosci.* **17**, 3303-3311.
- Mannaioni, G., Carla, V. and Moroni, F. (1996) Pharmacological characterization of metabotropic glutamate receptors potentiating NMDA responses in mouse cortical wedge preparations. *Br. J. Pharmac.* **118**, 1530-1536.
- Manzoni, O. and Bockaert, J. (1995) Metabotropic glutamate receptors inhibiting excitatory synapses in the CA1 area of rat hippocampus. *Eur. J. Neurosci.* **7**, 2518-2523.
- Manzoni, O. J., Weisskopf, M. G. and Nicoll, R. A. (1994) MCPG antagonizes metabotropic glutamate receptors but not long-term potentiation in the hippocampus. *Eur. J. Neurosci.* **6**, 1050-1054.
- Martin, L. J., Blackstone, C. D., Huganir, R. L. and Price, D. L. (1992) Cellular localization of a metabotropic glutamate receptor in rat brain. *Neuron* **9**, 259-270.
- Martin, S. J. and Morris, R. G. (1997) (R,S)-alpha-methyl-4-carboxyphenylglycine (MCPG) fails to block long-term potentiation under urethane anaesthesia in vivo. *Neuropharmacology* **36**, 1339-1354.
- Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R. and Nakanishi, S. (1991) Sequence and expression of a metabotropic glutamate receptor. *Nature* **349**, 760-765.
- Mattson, M. P., Cheng, B., Davis, D., Bryant, K., Lieberburg, I. and Rydel, R. E. (1992) beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J. Neurosci.* **12**, 376-389.
- Mayer, M. L. and Miller, R. J. (1990) Excitatory amino acid receptors, second messengers and regulation of intracellular Ca^{2+} in mammalian neurons. *Trends Pharmac. Sci.* **11**, 254-260.
- McBain, C. J., DiChiara, T. J. and Kauer, J. A. (1994) Activation of metabotropic glutamate receptors differentially affects two classes of hippocampal interneurons and potentiates excitatory synaptic transmission. *J. Neurosci.* **14**, 4433-4445.
- McCool, B. A., Pin, J. P., Harpold, M. M., Brust, P. F., Stauderman, K. A. and Lovinger, D. M. (1998) Rat group I metabotropic glutamate receptors inhibit neuronal Ca^{2+} channels via multiple signal transduction pathways in HEK 293 cells. *J. Neurophysiol.* **79**, 379-391.
- McCormick, D. A. and von Krosigk, M. (1992) Corticothalamic activation modulates thalamic firing through glutamate "metabotropic" receptors. *Proc. natl Acad. Sci. U.S.A.* **89**, 2774-2778.
- McDonald, J. W. and Schoepp, D. D. (1992) The metabotropic excitatory amino acid receptor agonist 1S,3R-ACPD selectively potentiates N-methyl-D-aspartate-induced brain injury. *Eur. J. Pharmac.* **215**, 353-354.
- McDonald, J. W., Fix, A. S., Tizzano, J. P. and Schoepp, D. D. (1993) Seizures and brain injury in neonatal rats induced by 1S,3R-ACPD, a metabotropic glutamate receptor agonist. *J. Neurosci.* **13**, 4445-4455.
- McGuinness, N., Anwyl, R. and Rowan, M. (1991) Trans-ACPD enhances long-term potentiation in the hippocampus. *Eur. J. Pharmac.* **197**, 231-232.
- McNamara, J. O. (1988) Pursuit of the mechanisms of kindling. *Trends Neurosci.* **11**, 33-36.
- Meldrum, B. S. (1994) The role of glutamate in epilepsy and other CNS disorders. *Neurology* **44**, S14-S23.
- Meltzer, L. T., Serpa, K. A. and Christoffersen, C. L. (1997) Metabotropic glutamate receptor-mediated inhibition and excitation of substantia nigra dopamine neurons. *Synapse* **26**, 184-193.
- Mendell, L. M. and Wall, P. D. (1965) Response of single dorsal horn cells to peripheral cutaneous unmyelinated fibers. *Nature* **206**, 97-99.
- de Mendonça, A. and Ribeiro, J. A. (1997) Contribution of metabotropic glutamate receptors to the depression of excitatory postsynaptic potentials during hypoxia. *Neuroreport* **8**, 3667-3671.
- Mercuri, N. B., Stratta, F., Calabresi, P., Bonci, A. and Bernardi, G. (1993) Activation of metabotropic glutamate receptors induces an inward current in rat dopamine mesencephalic neurons. *Neuroscience* **56**, 399-407.
- Merlin, L. R. and Wong, R. K. (1997) Role of group I metabotropic glutamate receptors in the patterning of epileptiform activities in vitro. *J. Neurophysiol.* **78**, 539-544.
- Merlin, L. R., Taylor, G. W. and Wong, R. K. (1995) Role of metabotropic glutamate receptor subtypes in the patterning of epileptiform activities in vitro. *J. Neurophysiol.* **74**, 896-900.
- Merlin, L. R., Bergold, P. J. and Wong, R. K. (1998) Requirement of protein synthesis for group I mGluR-mediated induction of epileptiform discharges. *J. Neurophysiol.* **80**, 989-993.
- Michaelis, E. K. (1998) Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. *Progr. Neurobiol.* **54**, 369-415.
- Moghaddam, B. and Adams, B. W. (1998) Reversal of phencyclidine effects by a group II metabotropic glutamate receptor agonist in rats [see comments]. *Science* **281**, 1349-1352.
- Monaghan, D. T., Bridges, R. J. and Cotman, C. W. (1989) The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. *A. Rev. Pharmac. Toxic.* **29**, 365-402.
- Moroni, F., Lombardi, G., Thomsen, C., Leonardi, P., Attucci, S., Peruginelli, F., Torregrossa, S. A., Pellegrini-Giampietro, D. E., Luneia, R. and Pellicciari, R. (1997) Pharmacological characterization of l-aminoinidan-1,5-dicarboxylic acid, a potent mGluR1 antagonist. *J. Pharmac. exp. Ther.* **281**, 721-729.
- Morris, R. G., Anderson, E., Lynch, G. S. and Baudry, M. (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature* **319**, 774-776.

- Morris, R. G. M. (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Meth.* 11, 47-60.
- Mukhin, A., Fan, L. and Faden, A. I. (1996) Activation of metabotropic glutamate receptor subtype mGluR1 contributes to post-traumatic neuronal injury. *J. Neurosci.* 16, 6012-6020.
- Mukhin, A. G., Ivanova, S. A. and Faden, A. I. (1997) mGluR modulation of post-traumatic neuronal death: role of NMDA receptors. *Neuroreport* 8, 2561-2566.
- Nakahara, K., Okada, M. and Nakanishi, S. (1997) The metabotropic glutamate receptor mGluR5 induces calcium oscillations in cultured astrocytes via protein kinase C phosphorylation. *J. Neurochem.* 69, 1467-1475.
- Nakanishi, S. (1994) Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity. *Neuron* 13, 1031-1037.
- Nakanishi, S. and Masu, M. (1994) Molecular diversity and functions of glutamate receptors. *A. Rev. Biophys. Biomolec. Struct.* 23, 319-348.
- Nakanishi, S., Masu, M., Bessho, Y., Nakajima, Y., Hayashi, Y. and Shigemoto, R. (1994) Molecular diversity of glutamate receptors and their physiological functions. *Exs* 71, 71-80.
- Nakanishi, S., Nakajima, Y., Masu, M., Ueda, Y., Nakahara, K., Watanabe, D., Yamaguchi, S., Kawabata, S. and Okada, M. (1998) Glutamate receptors: brain function and signal transduction. *Brain Res. Brain Res. Rev.* 26, 230-235.
- Neugebauer, V., Lucke, T. and Schaible, H. G. (1994) Requirement of metabotropic glutamate receptors for the generation of inflammation-evoked hyperexcitability in rat spinal cord neurons. *Eur. J. Neurosci.* 6, 1179-1186.
- Neugebauer, V., Keele, N. B. and Shinnick-Gallagher, P. (1997) Epileptogenesis in vivo enhances the sensitivity of inhibitory presynaptic metabotropic glutamate receptors in basolateral amygdala neurons in vitro. *J. Neurosci.* 17, 983-995.
- Nicoletti, F., Wroblewski, J. T., Novelli, A., Alho, H., Guidotti, A. and Costa, E. (1986) The activation of inositol phospholipid metabolism as a signal-transducing system for excitatory amino acids in primary cultures of cerebellar granule cells. *J. Neurosci.* 6, 1905-1911.
- Nicoletti, F., Bruno, V., Copani, A., Casabona, G. and Knopfel, T. (1996) Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? *Trends Neurosci.* 19, 267-271.
- Nicoll, R. A., Oliet, S. H. R. and Malenka, R. C. (1998) NMDA receptor-dependent and metabotropic glutamate receptor-dependent forms of long-term depression coexist in CA1 hippocampal pyramidal cells. *Neurobiol. Learn Mem.* 70, 62-72.
- Nielsen, K. S., Macphail, E. M. and Riedel, G. (1997) Class I mGlu receptor antagonist 1-aminoinidan-1,5-dicarboxylic acid blocks contextual but not cue conditioning in rats. *Eur. J. Pharmacol.* 326, 105-108.
- Nusser, Z., Mulvihill, E., Streit, P. and Somogyi, P. (1994) Subsynaptic segregation of metabotropic and ionotropic glutamate receptors as revealed by immunogold localization. *Neuroscience* 61, 421-427.
- Nusser, Z., Roberts, J. D., Baude, A., Richards, J. G., Sieghart, W. and Somogyi, P. (1995) Immunocytochemical localization of the alpha 1 and beta 2/3 subunits of the GABAA receptor in relation to specific GABAergic synapses in the dentate gyrus. *Eur. J. Neurosci.* 7, 630-646.
- O'Connor, J. J., Rowan, M. J. and Anwyl, R. (1994) Long-lasting enhancement of NMDA receptor-mediated synaptic transmission by metabotropic glutamate receptor activation. *Nature* 367, 557-559.
- Ohishi, H., Shigemoto, R., Nakanishi, S. and Mizuno, N. (1993) Distribution of the messenger RNA for a metabotropic glutamate receptor, mGluR2, in the central nervous system of the rat. *Neuroscience* 53, 1009-1018.
- Ohno, M. and Watanabe, S. (1996) Concurrent blockade of hippocampal metabotropic glutamate and N-methyl-D-aspartate receptors disrupts working memory in the rat. *Neuroscience* 70, 303-311.
- Ohno, M. and Watanabe, S. (1998) Enhanced N-methyl-D-aspartate function reverses working memory failure induced by blockade of group I metabotropic glutamate receptors in the rat hippocampus. *Neurosci. Lett.* 240, 37-40.
- Oliet, S. H., Malenka, R. C. and Nicoll, R. A. (1997) Two distinct forms of long-term depression coexist in CA1 hippocampal pyramidal cells. *Neuron* 18, 969-982.
- Olney, J. W. (1994) Neurotoxicity of NMDA receptor antagonists: an overview. *Psychopharmac. Bull.* 30, 533-540.
- Olney, J. W. and Farber, N. B. (1995) Glutamate receptor dysfunction and schizophrenia. *Arch. Gen. Psychiat.* 52, 998-1007.
- Olney, J. W., Labruyere, J. and Price, M. T. (1989) Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs [see comments]. *Science* 244, 1360-1362.
- O'Mara, S. M., Rowan, M. J. and Anwyl, R. (1995) Metabotropic glutamate receptor-induced homosynaptic long-term depression and depotentiation in the dentate gyrus of the rat hippocampus in vitro. *Neuropharmacology* 34, 983-989.
- Opitz, T., Richter, P. and Reymann, K. G. (1994) The metabotropic glutamate receptor antagonist (+)-alpha-methyl-4-carboxyphenylglycine protects hippocampal CA1 neurons of the rat from in vitro hypoxia/hypoglycemia. *Neuropharmacology* 33, 715-717.
- Opitz, T., Richter, P., Carter, A. J., Kozikowski, A. P., Shinozaki, H. and Reymann, K. G. (1995) Metabotropic glutamate receptor subtypes differentially influence neuronal recovery from in vitro hypoxia/hypoglycemia in rat hippocampal slices. *Neuroscience* 68, 989-1001.
- Orlando, L. R., Standaert, D. G., Penney, J. B., Jr. and Young, A. B. (1995) Metabotropic receptors in excitotoxicity: (S)-4-carboxy-3-hydroxyphenylglycine ((S)-4C3HPG) protects against rat striatal quinolinic acid lesions. *Neurosci. Lett.* 202, 109-112.
- Otani, S. and Ben-Ari, Y. (1991) Metabotropic receptor-mediated long-term potentiation in rat hippocampal slices. *Eur. J. Pharmacol.* 205, 325-326.
- Otani, S., Ben-Ari, Y. and Roisin-Lallemand, M. P. (1993) Metabotropic receptor stimulation coupled to weak tetanus leads to long-term potentiation and a rapid elevation of cytosolic protein kinase C activity. *Brain Res.* 613, 1-9.
- Overstreet, L. S., Pasternak, J. F., Colley, P. A., Slater, N. T. and Trommer, B. L. (1997) Metabotropic glutamate receptor mediated long-term depression in developing hippocampus. *Neuropharmacology* 36, 831-844.
- Palmer, E., Monaghan, D. T. and Cotman, C. W. (1989) Trans-ACPD, a selective agonist of the phosphoinositide-coupled excitatory amino acid receptor. *Eur. J. Pharmacol.* 166, 585-587.
- Palmer, M. J., Irving, A. J., Seabrook, G. R., Jane, D. E. and Collingridge, G. L. (1997) The group I mGlu receptor agonist DHPG induces a novel form of LTD in the CA1 region of the hippocampus. *Neuropharmacology* 36, 1517-1532.
- Paolillo, M., Montecucco, A., Zanassi, P. and Schinelli, S. (1998) Potentiation of dopamine-induced cAMP formation by group I metabotropic glutamate receptors via protein kinase C in cultured striatal neurons [In Process Citation]. *Eur. J. Neurosci.* 10, 1937-1945.
- Patel, S., Chapman, A. G., Graham, J. L., Meldrum, B. S. and Frey, P. (1990) Anticonvulsant activity of the NMDA antagonists, D-(-)-4-(3-phosphonopropyl) piperazine-2-carboxylic acid (D-CPP) and D-(-)-4-(3-phosphonoprop-2-enyl) piperazine-2-carboxylic acid (D-CPPene) in a rodent and a primate model of reflex epilepsy. *Epilepsy Res.* 7, 3-10.
- Pellicciari, R., Luneia, R., Costantino, G., Marinozzi, M., Natalini, B., Jakobsen, P., Kanstrup, A., Lombardi, G., Moroni, F. and Thomsen, C. (1995) 1-Aminoinidan-1,5-dicarboxylic acid: a novel antagonist at phospholipase C-linked metabotropic glutamate receptors. *J. Med. Chem.* 38, 2874-2876.
- Pellicciari, R., Raimondo, M., Marinozzi, M., Natalini, B., Costantino, G. and Thomsen, C. (1996) (S)-(+)-2-(3'-carboxy-bicyclo[1.1.1]pentyl)-glycine, a structurally new group I metabotropic glutamate receptor antagonist. *J. Med. Chem.* 39, 2874-2876.
- Phillips, R. G. and LeDoux, J. E. (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav. Neurosci.* 106, 274-285.
- Pile, A., Branski, P., Palucha, A., Tokarski, K. and Bijak, M. (1998) Antidepressant treatment influences group I of glutamate metabotropic receptors in slices from hippocampal CA1 region. *Eur. J. Pharmacol.* 349, 83-87.
- Pin, J.-P. and Duvoisin, R. (1995) The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* 34, 1-26.
- Pisani, A., Culabresi, P., Centonze, D. and Bernardi, G. (1997) Enhancement of NMDA responses by group I metabotropic glutamate receptor activation in striatal neurons. *Br. J. Pharmacol.* 120, 1007-1014.
- Pizzi, M., Fallacara, C., Arrighi, V., Memo, M. and Spano, P. F. (1993) Attenuation of excitatory amino acid toxicity by metabotropic glutamate receptor agonists and aniracetam in primary cultures of cerebellar granule cells. *J. Neurochem.* 61, 683-689.
- Poncer, J. C., Shinozaki, H. and Miles, R. (1995) Dual modulation of synaptic inhibition by distinct metabotropic glutamate receptors in the rat hippocampus. *J. Physiol. (Lond.)* 485, 121-134.

- Pook, P. C.-K., Sunter, D. C., Udvarhelyi, P. M. and Watkins, J. C. (1992) Evidence for presynaptic depression of monosynaptic excitation in neonatal rat motoneurons by (1S,3S)- and (1S,3R)-ACPD. *Exp. Physiol.* **77**, 529–532.
- Pulsinelli, W. A. (1985) Selective neuronal vulnerability: morphological and molecular characteristics. *Progr. Brain Res.* **63**, 29–37.
- Raff, M. C., Barres, B. A., Burne, J. F., Coles, H. S., Ishizaki, Y. and Jacobson, M. D. (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* **262**, 695–700.
- Rahman, S. and Neuman, R. S. (1996) Characterization of metabotropic glutamate receptor-mediated facilitation of N-methyl-D-aspartate depolarization of neocortical neurones. *Br. J. Pharmacol.* **117**, 675–683.
- Richter-Levin, G., Errington, M. L., Maegawa, H. and Bliss, T. V. (1994) Activation of metabotropic glutamate receptors is necessary for long-term potentiation in the dentate gyrus and for spatial learning. *Neuropharmacology* **33**, 853–857.
- Rickard, N. S. and Ng, K. T. (1995) Blockade of metabotropic glutamate receptors prevents long-term memory consolidation. *Brain Res. Bull.* **36**, 355–359.
- Riedel, G. (1996) Function of metabotropic glutamate receptors in learning and memory. *Trends Neurosci.* **19**, 219–224.
- Riedel, G. and Reymann, K. G. (1996) Metabotropic glutamate receptors in hippocampal long-term potentiation and learning and memory. *Acta Physiol. Scand.* **157**, 1–19.
- Riedel, G., Wetzel, W. and Reymann, K. G. (1994) (R,S)-alpha-methyl-4-carboxyphenylglycine (MCPG) blocks spatial learning in rats and long-term potentiation in the dentate gyrus in vivo. *Neurosci. Lett.* **167**, 141–144.
- Riedel, G., Casabona, G. and Reymann, K. G. (1995) Inhibition of long-term potentiation in the dentate gyrus of freely moving rats by the metabotropic glutamate receptor antagonist MCPG. *J. Neurosci.* **15**, 87–98.
- Roberts, P. J. (1995) Pharmacological tools for the investigation of metabotropic glutamate receptors (mGluRs): phenylglycine derivatives and other selective antagonists—an update. *Neuropharmacology* **34**, 813–819.
- Roche, K. W., Tingley, W. G. and Huganir, R. L. (1994) Glutamate receptor phosphorylation and synaptic plasticity. *Curr. Opin. Neurobiol.* **4**, 383–388.
- Romano, C., Sesma, M. A., McDonald, C. T., O'Malley, K., Van den Pol, A. N. and Olney, J. W. (1995) Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. *J. Comp. Neurol.* **355**, 455–469.
- Rothman, S. M. and Olney, J. W. (1986) Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Ann. Neurol.* **19**, 105–111.
- Rothman, S. M. and Olney, J. W. (1995) Excitotoxicity and the NMDA receptor—still lethal after eight years. *Trends Neurosci.* **18**, 57–58.
- Rutecki, P. A., Lebeda, F. J. and Johnston, D. (1987) 4-Aminopyridine produces epileptiform activity in hippocampus and enhances synaptic excitation and inhibition. *J. Neurophysiol.* **57**, 1911–1924.
- Sacaan, A. I. and Schoepp, D. D. (1992) Activation of hippocampal metabotropic excitatory amino acid receptors leads to seizures and neuronal damage. *Neurosci. Lett.* **139**, 77–82.
- Sacaan, A. I., Monn, J. A. and Schoepp, D. D. (1991) Intrastriatal injection of a selective metabotropic excitatory amino acid receptor agonist induces contralateral turning in the rat. *J. Pharmacol. exp. Ther.* **259**, 1366–1370.
- Sacaan, A. I., Byrnaster, F. P. and Schoepp, D. D. (1992) Metabotropic glutamate receptor activation produces extrapyramidal motor system activation that is mediated by striatal dopamine. *J. Neurochem.* **59**, 245–251.
- Sagara, Y. and Schubert, D. (1998) The activation of metabotropic glutamate receptors protects nerve cells from oxidative stress. *J. Neurosci.* **18**, 6662–6671.
- Sahara, Y. and Westbrook, G. L. (1993) Modulation of calcium currents by a metabotropic glutamate receptor involves fast and slow kinetic components in cultured hippocampal neurons. *J. Neurosci.* **13**, 3041–3050.
- Salt, T. E. and Eaton, S. A. (1995) Modulation of sensory neurone excitatory and inhibitory responses in the ventrobasal thalamus by activation of metabotropic excitatory amino acid receptors. *Neuropharmacology* **34**, 1043–1051.
- Salt, T. E. and Turner, J. P. (1998) Reduction of sensory and metabotropic glutamate receptor responses in the thalamus by the novel metabotropic glutamate receptor-1-selective antagonist S-2-methyl-4-carboxy-phenylglycine. *Neuroscience* **85**, 655–658.
- Sayer, R. J., Schwindt, P. C. and Crill, W. E. (1992) Metabotropic glutamate receptor-mediated suppression of L-type calcium current in acutely isolated neocortical neurons. *J. Neurophysiol.* **68**, 833–842.
- Schoepp, D., Bockaert, J. and Sladeczek, F. (1990) Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. *Trends Pharmacol. Sci.* **11**, 508–515.
- Schoepp, D. D., Goldsworthy, J., Johnson, B. G., Salhoff, C. R. and Baker, S. R. (1994) 3,5-Dihydroxyphenylglycine is a highly selective agonist for phosphoinositide-linked metabotropic glutamate receptors in the rat hippocampus. *J. Neurochem.* **63**, 769–772.
- Schoepp, D. D., Tizzano, J. P., Wright, R. A. and Fix, A. S. (1995) Reversible and irreversible neuronal injury induced by intrahippocampal infusion of the mGluR agonist 1S,3R-ACPD in the rat. *Neurodegeneration* **4**, 71–80.
- Schrader, L. A. and Tasker, J. G. (1997) Modulation of multiple potassium currents by metabotropic glutamate receptors in neurons of the hypothalamic supraoptic nucleus. *J. Neurophysiol.* **78**, 3428–3437.
- Schwarzer, C. and Sperk, G. (1998) Glutamate-stimulated neuropeptide Y mRNA expression in the rat dentate gyrus: a prominent role of metabotropic glutamate receptors. *Hippocampus* **8**, 274–288.
- Schwarzer, C., Kofler, N. and Sperk, G. (1998) Up-regulation of neuropeptide Y-Y2 receptors in an animal model of temporal lobe epilepsy. *Molec. Pharmacol.* **53**, 6–13.
- Selig, D. K., Lee, H. K., Bear, M. F. and Malenka, R. C. (1995) Reexamination of the effects of MCPG on hippocampal LTP, LTD, and depotentiation. *J. Neurophysiol.* **74**, 1075–1082.
- Sergueeva, O. A., Fedorov, N. B. and Reymann, K. G. (1993) An antagonist of glutamate metabotropic receptors, (R,S)-alpha-methyl-4-carboxyphenylglycine, prevents the LTP-related increase in postsynaptic AMPA sensitivity in hippocampal slices. *Neuropharmacology* **32**, 933–935.
- Shen, H., Gorter, J. A., Aronica, E., Zheng, X., Zhang, L., Bennett, M. V. and Zukin, R. S. (1995) Potentiation of NMDA responses by metabotropic receptor activation depends on NMDA receptor subunit composition. In: *Society for Neuroscience Abstracts*, Vol. 21 Part 1, p. 77.
- Shen, K. Z. and Johnson, S. W. (1997) A slow excitatory postsynaptic current mediated by G-protein-coupled metabotropic glutamate receptors in rat ventral tegmental dopamine neurons. *Eur. J. Neurosci.* **9**, 48–54.
- Shigemoto, R., Nakanishi, S. and Mizuno, N. (1992) Distribution of the mRNA for a metabotropic glutamate receptor (mGluR1) in the central nervous system: an in situ hybridization study in adult and developing rat. *J. comp. Neurol.* **322**, 121–135.
- Shigemoto, R., Nomura, S., Ohishi, H., Sugihara, H., Nakanishi, S. and Mizuno, N. (1993) Immunohistochemical localization of a metabotropic glutamate receptor, mGluR5, in the rat brain. *Neurosci. Lett.* **163**, 53–57.
- Shigemoto, R., Abe, T., Nomura, S., Nakanishi, S. and Hirano, T. (1994) Antibodies inactivating mGluR1 metabotropic glutamate receptor block long-term depression in cultured Purkinje cells. *Neuron* **12**, 1245–1255.
- Siliprandi, R., Lipartiti, M., Fadda, E., Sautter, J. and Manev, H. (1992) Activation of the glutamate metabotropic receptor protects retina against N-methyl-D-aspartate toxicity. *Eur. J. Pharmacol.* **219**, 173–174.
- Simon, R. P., Swan, J. H., Griffiths, T. and Meldrum, B. S. (1984) Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science* **226**, 850–852.
- Skolnick, P., Laver, R. T., Popik, P., Nowak, G., Paul, I. A. and Trullas, R. (1996) Adaptation of N-methyl-D-aspartate (NMDA) receptors following antidepressant treatment: implications for the pharmacotherapy of depression. *Pharmacopsychiatry* **29**, 23–26.
- Sladeczek, F., Pin, J. P., Recasens, M., Bockaert, J. and Weiss, S. (1985) Glutamate stimulates inositol phosphate formation in striatal neurones. *Nature* **317**, 717–719.
- Stefani, A., Pisani, A., Mercuri, N. B., Bernardi, G. and Calabresi, P. (1994) Activation of metabotropic glutamate receptors inhibits calcium currents and GABA-mediated synaptic potentials in striatal neurons. *J. Neurosci.* **14**, 6734–6743.
- Stefani, A., Pisani, A., Mercuri, N. B. and Calabresi, P. (1996) The modulation of calcium currents by the activation of mGluRs. Functional implications. *Molec. Neurobiol.* **13**, 81–95.
- Strasser, U., Lobner, D., Behrens, M. M., Canzoniero, L. M. and Choi, D. W. (1998) Antagonists for group I mGluRs attenuate

- excitotoxic neuronal death in cortical cultures [In Process Citation]. *Eur. J. Neurosci.* 10, 2848–2855.
- Sugiyama, H., Ito, I. and Hirono, C. (1987) A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature* 325, 531–533.
- Suzuki, K., Mori, N., Kittaka, H., Iwata, Y., Yamada, Y., Osonoe, K. and Niwa, S. (1996) Anticonvulsant action of metabotropic glutamate receptor agonists in kindled amygdala of rats. *Neurosci. Lett.* 204, 41–44.
- Swartz, K. J. and Bean, B. P. (1992) Inhibition of calcium channels in rat CA3 pyramidal neurons by a metabotropic glutamate receptor. *J. Neurosci.* 12, 4358–4371.
- Swartz, K. J., Merritt, A., Bean, B. P. and Lovinger, D. M. (1993) Protein kinase C modulates glutamate receptor inhibition of Ca^{2+} channels and synaptic transmission. *Nature* 361, 165–168.
- Tanabe, Y., Masu, M., Ishii, T., Shigemoto, R. and Nakanishi, S. (1992) A family of metabotropic glutamate receptors. *Neuron* 8, 169–179.
- Tang, E., Yip, P. K., Chapman, A. G., Jane, D. E. and Meldrum, B. S. (1997) Prolonged anticonvulsant action of glutamate metabotropic receptor agonists in inferior colliculus of genetically epilepsy-prone rats. *Eur. J. Pharmacol.* 327, 109–115.
- Thomas, M. J. and O'Dell, T. J. (1995) The molecular switch hypothesis fails to explain the inconsistent effects of the metabotropic glutamate receptor antagonist MCPG on long-term potentiation. *Brain Res.* 695, 45–52.
- Thompson, S. W., Gerber, G., Sivilotti, L. G. and Woolf, C. J. (1992) Long duration ventral root potentials in the neonatal rat spinal cord in vitro; the effects of ionotropic and metabotropic excitatory amino acid receptor antagonists. *Brain Res.* 595, 87–97.
- Thompson, S. W., Woolf, C. J. and Sivilotti, L. G. (1993) Small-caliber afferent inputs produce a heterosynaptic facilitation of the synaptic responses evoked by primary afferent A-fibers in the neonatal rat spinal cord in vitro. *J. Neurophysiol.* 69, 2116–2128.
- Thomsen, C., Klitgaard, H., Sheardown, M., Jackson, H. C., Eskesen, K., Jacobsen, P., Treppendahl, S. and Suzdak, P. D. (1994) (S)-4-carboxy-3-hydroxyphenylglycine, an antagonist of metabotropic glutamate receptor (mGluR) 1a and an agonist of mGluR2, protects against audiogenic seizures in DBA/2 mice. *J. Neurochem.* 62, 2492–2495.
- Tizzano, J. P., Griffey, K. I., Johnson, J. A., Fix, A. S., Helton, D. R. and Schoepp, D. D. (1993) Intracerebral 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) produces limbic seizures that are not blocked by ionotropic glutamate receptor antagonists. *Neurosci. Lett.* 162, 12–16.
- Tizzano, J. P., Griffey, K. I. and Schoepp, D. D. (1995) Induction or protection of limbic seizures in mice by mGluR subtype selective agonists. *Neuropharmacology* 34, 1063–1067.
- Toms, N. J., Jane, D. E., Tse, H. W. and Roberts, P. J. (1995) Characterization of metabotropic glutamate receptor-stimulated phosphoinositide hydrolysis in rat cultured cerebellar granule cells. *Br. J. Pharmacol.* 116, 2824–2827.
- Trujillo, K. A. and Akil, H. (1991) Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science* 251, 85–87.
- Trullas, R. and Sholnick, P. (1990) Functional antagonists at the NMDA receptor complex exhibit antidepressant actions. *Eur. J. Pharmacol.* 185, 1–10.
- Turski, L., Bressler, K., Rettig, K. J., Loschmann, P. A. and Wachtel, H. (1991) Protection of substantia nigra from MPP⁺ neurotoxicity by N-methyl-D-aspartate antagonists [see comments]. *Nature* 349, 414–418.
- Ugolini, A., Corsi, M. and Bordini, F. (1997) Potentiation of NMDA and AMPA responses by group I mGluR in spinal cord motoneurons. *Neuropharmacology* 36, 1047–1055.
- Valerio, A., Paterlini, M., Boifava, M., Memo, M. and Spano, P. (1997a) Metabotropic glutamate receptor mRNA expression in rat spinal cord. *Neuroreport* 8, 2695–2699.
- Valerio, A., Rizzonelli, P., Paterlini, M., Moretto, G., Knöpfel, T., Kuhn, R., Memo, M. and Spano, P. (1997b) mGluR5 metabotropic glutamate receptor distribution in rat and human spinal cord: a developmental study. *Neurosci. Res.* 28, 49–57.
- Verkhratsky, A. and Kettenmann, H. (1996) Calcium signalling in glial cells. *Trends Neurosci.* 19, 346–352.
- Vickery, R. M., Morris, S. H. and Bindman, L. J. (1997) Metabotropic glutamate receptors are involved in long-term potentiation in isolated slices of rat medial frontal cortex. *J. Neurophysiol.* 78, 3039–3046.
- Vidnyánszky, Z., Hámosi, J., Négyessy, L., Rüegg, D., Knöpfel, T., Kuhn, R. and Görcs, T. J. (1994) Cellular and subcellular localization of the mGluR1a metabotropic glutamate receptor in rat spinal cord. *NeuroReport* 6, 209–213.
- Wang, X. F. and Daw, N. W. (1996) Metabotropic glutamate receptors potentiate responses to NMDA and AMPA from layer V cells in rat visual cortex. *J. Neurophysiol.* 76, 808–815.
- Wang, Y., Rowan, M. J. and Anwyl, R. (1995) (RS)-alpha-Methyl-4-carboxyphenylglycine inhibits long-term potentiation only following the application of low frequency stimulation in the rat dentate gyrus in vitro. *Neurosci. Lett.* 197, 207–210.
- Watkins, J. and Collingridge, G. (1994) Phenylglycine derivatives as antagonists of metabotropic glutamate receptors. *Trends Pharmacol. Sci.* 15, 333–342.
- Wigmore, M. A. and Lacey, M. G. (1998) Metabotropic glutamate receptors depress glutamate-mediated synaptic input to rat mid-brain dopamine neurones in vitro. *Br. J. Pharmacol.* 123, 667–674.
- Willis, W. D. and Westlund, K. N. (1997) Neuroanatomy of the pain system and of the pathways that modulate pain. *J. Clin. Neurophysiol.* 14, 2–31.
- Wilsch, V. W., Behnisch, T., Jäger, T., Reymann, K. G. and Balschun, D. (1998) When are class I metabotropic glutamate receptors necessary for long-term potentiation? *J. Neurosci.* 18, 6071–6080.
- Womble, M. D. and Moises, H. C. (1994) Metabotropic glutamate receptor agonist ACPD inhibits some, but not all, muscarinic-sensitive K^{+} conductances in basolateral amygdaloid neurons. *Synapse* 17, 69–75.
- Woolf, C. J. (1983) Evidence for a central component of post-injury pain hypersensitivity. *Nature* 306, 686–688.
- Woolf, C. J. and Wall, P. D. (1986) Relative effectiveness of C primary afferent fibers of different origins in evoking a prolonged facilitation of the flexor reflex in the rat. *J. Neurosci.* 6, 1433–1442.
- Yamamoto, T. and Yaksh, T. L. (1992) Comparison of the antinociceptive effects of pre- and posttreatment with intrathecal morphine and MK801, an NMDA antagonist, on the formalin test in the rat. *Anesthesiology* 77, 757–763.
- Young, M. R., Fleetwood-Walker, S. M., Mitchell, R. and Munro, F. E. (1994) Evidence for a role of metabotropic glutamate receptors in sustained nociceptive inputs to rat dorsal horn neurons. *Neuropharmacology* 33, 141–144.
- Young, M. R., Fleetwood-Walker, S. M., Mitchell, R. and Dickinson, T. (1995) The involvement of metabotropic glutamate receptors and their intracellular signalling pathways in sustained nociceptive transmission in rat dorsal horn neurons. *Neuropharmacology* 34, 1033–1041.
- Young, M. R., Fleetwood-Walker, S. M., Dickinson, T., Blackburn-Munro, G., Sparrow, H., Birch, P. J. and Bountra, C. (1997) Behavioural and electrophysiological evidence supporting a role for group I metabotropic glutamate receptors in the mediation of nociceptive inputs to the rat spinal cord. *Brain Res.* 777, 161–169.
- Yu, S. P., Sensi, S. L., Canzoniero, L. M., Buisson, A. and Choi, D. W. (1997) Membrane-delimited modulation of NMDA currents by metabotropic glutamate receptor subtypes 1/5 in cultured mouse cortical neurons. *J. Physiol. (Lond.)* 499, 721–732.

ATCC

10801 University Blvd • Manassas, VA 20110-2209 • Telephone: 703-365-2700 • FAX: 703-365-2745

**BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE**

INTERNATIONAL FORM

**RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2**

To: (Name and Address of Depositor or Attorney)

Genetics Institute
Attn: Janet Paulsen
87 Cambridge Park Drive
Cambridge, MA 02140

Deposited on Behalf of: Genetics Institute

Identification Reference by Depositor:
cDNA Clone (Human): Y1176

Patent Deposit Designation
PTA-2775

The deposit was accompanied by: ☐ a scientific description ☐ a proposed taxonomic description indicated above.

The deposit was received December 12, 2000 by this International Depository Authority and has been accepted.

AT YOUR REQUEST: ☒ We will inform you of requests for the strain for 30 years.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

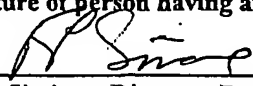
If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested December 27, 2000. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:



Frank Simione, Director, Patent Depository

Date: January 9, 2001

cc: Andrea Ryan (Ref: Docket or Case No.: AM100369)

Docket No: AM100369
Patent

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re of Application of: Brian Gaither BATES *et al.*
Application No.: 10/027,923 Group No.: 1647
Filed: December 21, 2001 Examiner: Turner, Sharon L
For: NOVEL GLUTAMATE RECEPTOR MODULATORY PROTEINS AND
NUCLEIC ACID MOLECULES AND USES THEREFOR
Confirmation No.: 9899
Customer Number: 25291

Mail Stop Non-Fee Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

CERTIFICATE OF MAILING 37 CFR §1.10

I hereby certify that this paper and the documents referred to as enclosed therein are being deposited with the United States Postal Service on the date written below in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EV295518632US addressed to the Mail Stop Non-Fee Amendment, Commissioner for Patents, PO Box 1450, Alexandria, VA 22313-1450.

Date

DECLARATION OF KAMALAKAR GULUKOTA
UNDER 37 C.F.R. § 1.132

Dear Sir:

I, Kamalakar Gulukota, declare the following in support of the above-captioned application:

1. I am the Kamalakar Gulukota named as an inventor in the above-captioned application. I am also the Kamalakar Gulukota named as an inventor in U.S. Patent Application Publication US 2002/0142952 (Wong *et al.*). I am a citizen of India, presently residing at 16-2-836/D/A/2, Madhav Nagar, Saidabad, Hyderabad 500 059, India.

2. I hold a Ph.D. from the University of Illinois at Urbana Champaign in Biophysics with a major emphasis in the area of Protein Folding. I am currently a Head, Clinical R&D in GVK Biosciences Private Limited where I head up the clinical research of the company. I was previously an employee of Wyeth, where I worked on sequence analysis and other bioinformatics activities in the genomics area.

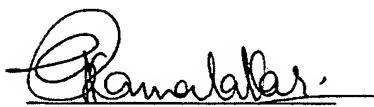
3. I have read the Office Action mailed on February 10, 2004, in connection with the above-captioned application. It is my understanding, based on my review of the action, that the Examiner in charge of examining this application has advanced a rejection based on a lack of novelty for the presently claimed invention, in view of the Wong *et al.* published application.

4. I performed the nucleic acid sequence analysis that identified the genomic and open reading frame sequences (e.g., SEQ ID NO:61) set forth in the Wong *et al.* U.S. Patent Application Publication No. US 2002/0142952. I also performed the BLAST sequence analysis and characterization of the polynucleotide sequence (SEQ ID NO:1) and the encoded protein sequence (SEQ ID NO:2) described and set forth in the present application as mGluR5M (U.S. Application No. 10/027,923).

5. The referenced U.S. Patent Application Publication No. US 2002/0142952 is not by another.

6. This work was performed in the U.S. during my employment at Wyeth.

7. All statements made in this declaration of my own knowledge are true and all statements made in this declaration on information and belief are believed to be true, with the understanding that knowledge of these statements being willfully false and the like is punishable by fine or imprisonment or both, under 18 U.S.C. § 1001 and may jeopardize the validity of any patent to be obtained from the referenced application.


Kamalakar Gulukota

06 May 2004
Date